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Vol. 11. No. 2. JUNE, 1956

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BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY

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LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, W.C.1

YEARLY SUBSCRIPTION (4 NUMBERS) £4 4s. U.S.A. $13.50 SINGLE NUMBERS £1 5s.

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Brit. J. Pharmacol. (1956), **11**, 93.

OBSERVATIONS ON A SUBSTANCE IN HUMAN PLASMA WHICH GIVES A SLOW CONTRACTION OF GUINEA-PIG GUT *IN VITRO*

BY

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(RECEIVED APRIL 5, 1955)

It has been known for many years that serum and plasma stimulate smooth muscle. This property of serum has been recently attributed to 5-hydroxytryptamine (Rapport, 1949). The vasoconstrictor and muscle stimulating properties of plasma and of serum were attributed by Stewart and Zucker (1913) to a change that takes place in shed blood. These properties are not developed —at least not fully—with the change of fibrinogen to fibrin, since the constrictor action of different plasmas (citrate, peptone or hirudin plasma) differs greatly, whereas the absence of coagulation is common to all.

The G2 fraction of human plasma causes a slow contraction of the isolated guinea-pig gut *in vitro*. The same effect has been observed with human blood plasma. Previous investigators (O'Connor, 1912; Trendelenburg, 1911; and Kahn, 1912) showed that the smooth muscle stimulating property of serum is, in general, greater than that of plasma. If this effect, as has been suggested, arises from preliminary stages in coagulation, or breakdown of platelets, it may still occur in non-coagulable blood, for the coagulation process may be interrupted at different points. The prevention of clotting has little influence on the development of the smooth muscle stimulating property. Coagulation of blood, from which the G2 fraction has been obtained, can be prevented by the addition of trisodium citrate. As the cellular elements then rapidly disintegrate it is probable that the preliminary stages of coagulation occur. There is, therefore, no reason to believe that the slow muscle contractor substance (SCS) in the G2 fraction from plasma will be chemically different from that in serum.

This paper describes the isolation and chemical nature of SCS from the G2 fraction of human blood plasma. A structure is proposed for a fatty acid which was isolated and which may be responsible for the effect on guinea-pig jejunum.

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METHODS

Chemical

Preparation of the G2 Fraction from Human Blood Plasma.—The G2 fraction was precipitated from human blood plasma by the ether method of Kekwick and Mackay (1948) after the separation of fibrinogen and prothrombin.

Isolation of SCS from the G2 Fraction.—The methods of purification involved extractions with organic solvents or redistilled water only. The alumina (Savory and Moore) used for chromatographic analysis of SCS yielded some inorganic impurities when aqueous methanol was the solvent, but these were insoluble in absolute methanol and redistilled ether, which were therefore used for the further purification of the active material.

The isolation consisted of five steps :

Step 1. *Extraction with Aqueous Ethanol.*—The G2 material (dry wt. 120 g.) precipitated from 13.5 l. of human plasma was suspended in 0.85% saline (1.2 l.), and N-sodium hydroxide added dropwise to *p*H 7.3. After the addition of 1.8 l. of 96% ethanol, the mixture was thoroughly stirred and left at room temperature overnight. The protein precipitate was removed by centrifugation and filtration and the filtrate evaporated below 40° C. and under reduced pressure to give 20–30 ml. of a fatty emulsion. Octan-2-ol was added to control foaming. The dried extract weighed 7.2 g., 100 units/g. (one unit is the activity of 50 mg. G2; see Table I).

Step 2. *Removal of Material Insoluble in Acetone.*—To the concentrate thus obtained, 10 vol. acetone were added. The mixture was shaken, stirred at room temperature for 30 min. and kept at 1–2° C. overnight. The insoluble material (dry wt. 4.2 g., no activity detected) was removed and the clear, light yellow supernatant solution evaporated to dryness under reduced pressure and below 40° C. The wet residue was partly dehydrated by recovering it from solution in butanol, by distillation *in vacuo* and by storing over P_2O_5 *in vacuo*. The dry solid (dry wt. 4.8 g., 200

units/g.) was dissolved in about 2 ml. of absolute methanol containing 10% anhydrous acetone; any insoluble material which formed was removed by centrifugation.

Step 3. *Chromatography.*—The material obtained in step 2 was applied to a column (Savory and Moore) of alumina (25 × 1.5 cm.) previously washed with 100 ml. absolute methanol containing 10% acetone, and developed with 200 ml. of the same solvent mixture. This eluate when dried had an activity of 1,000 units/g. The active substance was eluted from the column by 50% aqueous methanol, the first 80 ml. of the eluate being discarded. The following 150 ml. of effluent was evaporated to dryness under reduced pressure and the solid obtained was extracted with a few ml. absolute methanol. About 50 mg. active material (20,000 units/g.) was obtained.

Step 4. *Acetic Acid Precipitation.*—The material (step 3) was dissolved in 5 ml. warm distilled water. Glacial acetic acid (AR, 0.1 ml.) was added with stirring, and the copious white precipitate that formed was left in the cold for 1 hr. to form a coherent mat. The insoluble material was separated by filtration and centrifugation, washed with 1 ml. portions of 2% acetic acid, and finally removed from the filter paper with 5 ml. hot absolute methanol. A yellowish, transparent, semi-crystalline substance was obtained (28 mg., 20,000 units/g.).

TABLE I

THE CONCENTRATION OF SCS IN, AND ITS ISOLATION FROM, THE G2 FRACTION OF HUMAN PLASMA

Fraction	Recovery of SCS from G2 (U./g. G2)	U.*/g. Dry Weight
G2	20	20
60% alcohol extract	12	100
Acetone extract	9	200
50% methanol eluate	8	20,000
Acid precipitate	5	20,000
Crystalline substance	5	50,000

* One unit of SCS is defined as that quantity which causes, in the isolated guinea-pig jejunum suspended in a 10 ml. bath, a slow contraction similar to that obtained with 50 mg. of G2.

Step 5. *Ether Extraction.*—The acid precipitate (step 4) was dried over P_2O_5 and NaOH pellets and the material was extracted with 5 ml. peroxide-free ether. The colourless crystalline material obtained from the ether solution was then further purified by extraction with acetone in the cold (10% solution at −10° C.). The acetone soluble material was crystallized from peroxide-free ether to yield about 12 mg. of a colourless crystalline substance (m.p. 34° C., 50,000 units/g.).

This method of isolation resulted in more than a 1,000-fold concentration of the substance on a dry weight basis (Table I). The recovery of activity is about 25% of that of the original G2 fraction.

Biological Assays

Rabbit Jejunum.—The determinations on isolated rabbit jejunum were made in a Dale's apparatus in the usual way, using a chamber of 10 ml. capacity. Fig. 1 shows the effect of atropine on acetylcholine and G2 fraction responses. The G2 fraction or acetylcholine was injected into the organ bath 3 min. after atropine. With acetylcholine, the jejunum was washed directly after the contraction had reached its summit. With the G2 fraction the jejunum was washed 1 min. after injection. Note the slow relaxation of the jejunum, which took 5–8 min.

Guinea-pig Jejunum.—The determinations on isolated guinea-pig jejunum were made in a Dale apparatus in the usual way, using a chamber of 10 ml. capacity. Solutions of the unknown were diluted until 0.2 ml. gave a response identical with that of 0.2 ml. of a standard control solution of SCS. The standard was a solution of G2 fraction pooled from 5 runs. A concentration of 250 mg./ml. was arbitrarily designated as containing 1 unit of activity. Fig. 2 shows the effect of G acid (the crystalline SCS) on isolated guinea-pig jejunum. Note the slow contraction of the jejunum, which started 20–30 sec. after adding G acid to the organ bath. When the contraction reached its maximum 1 min. after injection, the organ bath was washed out. The contraction persisted after washing out the organ bath, slowly returning to the base line. This took 2–3 min.

Rabbit Coronaries.—The isolated rabbit heart was perfused via the aorta with Ringer Locke solution, the perfusate being collected and measured. The decrease in the rate of flow following injection of G2 fraction or G acid was taken as a measure of coronary constriction. The heart has not been used as a routine test.

Haemolysis.—The methods used to assay the haemolytic activity of G acid were essentially those used by Laser (1949) with vaccenic acid. Human erythrocytes obtained from defibrinated blood were used. A few ml. of erythrocytes were twice washed with 0.15 M-phosphate buffer (*p*H 7.3) and 5–10% (v/v) suspensions prepared. A given suspension was divided into several parts and a fresh one was used for each series to prevent protracted incubation at 37° C. before zero time. All experiments were done with 15 ml. test-tubes in a hot room at 37° C. The tubes were placed in a metal stand and viewed against a light source. End-points of haemolysis were reached when all cloudiness in the test-tubes had disappeared. The suspension medium was 0.15 M-phosphate buffer from Na_2HPO_4 and KH_2PO_4. A known amount of G acid was dissolved by warming in 0.32 N-NaOH. Stock solutions containing 0.5–1.0 mg./ml. were prepared. G acid and the erythrocyte suspension were separately incubated in test-tubes until they reached 37° C. G acid was then added to the test-tubes containing the medium, the erythrocytes being added last, at zero time. The contents of each test-tube were mixed by inverting the tube. To prevent a *p*H shift of the medium used for the measurement of haemolysis, 0.1 ml. 0.32 N-HCl was added for each 100 mg. of G acid dissolved in NaOH.

RESULTS

Chemical

Some Physical and Chemical Aspects of SCS.—The small amounts of SCS available prevented a detailed study of its physical and chemical properties. The isolation of derivatives or decomposition products was not attempted. However, ultra-violet absorption spectroscopy and paper chromatography helped to establish the purity and chemical nature of the substance.

Pure SCS oxidizes, and becomes inactive, easily. The material in the G2 fraction of plasma is probably protected by a stabilizer. A G2 fraction in saline kept at 1° C. for six months retained its activity unchanged, whereas a solution of the pure SCS can lose over 90% of its activity after one month under the same conditions.

The SCS was strongly acid, and the neutral equivalent, determined by the method of Fairbairn (1945), was 283. Since oxidation with potassium permanganate in acetone gave only one dicarboxylic acid (malonic acid), this value of 283 is considered as the molecular weight of a fatty acid. SCS will therefore subsequently be referred to as G acid. The iodine number of G acid, determined by the micro-method of Yasuda (1931), is 75. This relatively low value suggests that the double bond is close to the carboxyl group (theoretical 89.7 for one double bond in a C_{18} fatty acid). Freshly isolated G acid was used for chemical analysis, since old specimens give higher figures for the neutral equivalent value (theoretical 282) and low figures for the iodine value. This behaviour may be attributed to partial oxidation or polymerization of G acid. These changes were, however, suppressed by keeping the G acid in ampoules sealed in nitrogen.

Examination of surface film formed by this acid on a Langmuir trough suggested that: (a) It is a straight chain acid because the film compressed to 20 $Å^2$/molecule. Stenhagen (1940) has shown that disubstituted acetic acids occupy areas greater than 20 $Å^2$ (α-methyl, 29.5 $Å^2$). This film was on 0.01 N-HCl. (b) The film was also spread over 0.01 N-sulphuric acid containing 0.0025 N-permanganate. An expansion of the film resulted, and on compression the film behaved as if it were partly dissolving, which is what oleic acid does under similar conditions.

G acid has a characteristic ultra-violet absorption in the region 2,200–2,250 Å. The value of $E^{1\%}_{1\,cm.}$ in this region is 34.7. Similar ultra-violet absorption has been found for 2-octadecenoic acid in which the ethylenic bond is conjugated with the carbonyl double bond in the carboxyl group (Myers, 1951). In this instance, however, the amount of absorption is greater.

The amount of active hydrogen in G acid is 0.35% as determined by Drs. G. Weiler and F. B. Strauss. This value is compatible with the presence of one replaceable hydrogen atom in the molecule, that is, the carboxyl hydrogen atom. Presumably, no hydroxyl group is present. No keto groups could be identified, since no phenyl hydrazone derivative could be isolated. The probability of an epoxy mode of combination is rather remote, since no reaction took place with ammonia under pressure, and no amino acid was detected after the reaction (Swern and Findlay, 1952).

Oxidation of G acid with potassium permanganate in acetone gave one dicarboxylic acid, which moved on paper using an ethanol-ammonia-water mixture for irrigation at the same rate as an authentic sample of malonic acid (Long, Quale and Stedman, 1951), and a monocarboxylic acid which was indistinguishable on paper from pentadecylenic acid. It is probable, therefore, that the double bond in G acid occupies the 3–4 position in a C_{18} straight chain.

The criteria given below, together with the properties already described, lead one to believe that the isolated G acid is not grossly contaminated with other substances.

1. The absence of nitrogen eliminates the presence of bradykinin (Rocha e Silva, Beraldo, and Rosenfeld, 1949).

2. The failure to get biologically or chemically different fractions from the crystalline G acid by crystallization from acetone at low temperatures (Brown, 1941) suggests the absence of appreciable amounts of saturated fatty acids as contaminants.

3. The absence of higher unsaturated fatty acids (linoleic, linolenic and arachidonic acids) was indicated, since no distinct increase in ultra-violet absorption was detected in the regions 2,350 Å, 2,700 Å and 3,000 Å (Wiese and Hansen, 1953), after treatment of the G acid with alkali by the method of Hilditch, Morton, and Riley (1945).

4. The demonstration on paper of only one dicarboxylic acid after cleavage of the double bond with potassium permanganate in acetone solution excludes significant contamination by other isomeric unsaturated fatty acids.

Biological

Action on Guinea-pig Jejunum.—Guinea-pig jejunum is stimulated by the G2 fraction in a manner unlike that of histamine or acetylcholine. The G2 fraction, after a latent period of about 20 or 30 sec., produced a contraction which did not

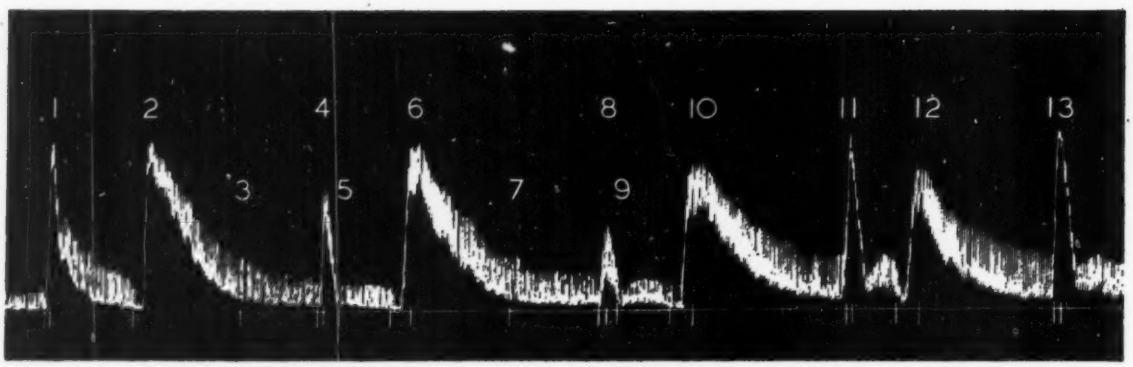

FIG. 1.—Rabbit jejunum. The effect of atropine on acetylcholine (ACh) and G2 fraction responses. (1) 1 μg. ACh. (2) 50 mg. G2 fraction. (3) 0.04 μg. atropine sulphate. (4) 1 μg. ACh. (5) 0.04 μg. atropine sulphate. (6) 50 mg. G2 fraction. (7) 0.1 μg. atropine sulphate. (8) 1 μg. ACh. (9) 0.1 μg. atropine sulphate. (10) 50 mg. G2 fraction. (11) 1 μg. ACh. (12) 50 mg. G2 fraction. (13) 1 μg. ACh.

reach its maximum for 1–2 min. Longer or shorter latent periods were sometimes observed, however, and, indeed, totally unresponsive preparations were occasionally encountered. Other characteristic features were that the contraction persisted after washing out the bath and that relaxation was slow. The effect of G2 fraction on smooth muscle was not antagonized by antazoline or by atropine. Rabbit jejunum behaved in a similar manner (Fig. 1).

The G acid produced a slow contraction similar to the G2 fraction. In a 10 ml. bath, 50 μg. G acid gave a response similar to 50 mg. G2 fraction on this preparation except that with the former the latent period was less (Fig. 2).

Other smooth muscle preparations have been used from time to time to investigate the biological aspects of the problem. The response of the isolated rabbit jejunum to the G2 fraction suggests that the active principle is not histamine. G2 fraction had no effect on isolated rat colon. This result rules out the presence of appreciable amounts of many pharmacological substances (for example, acetylcholine or 5-hydroxytryptamine).

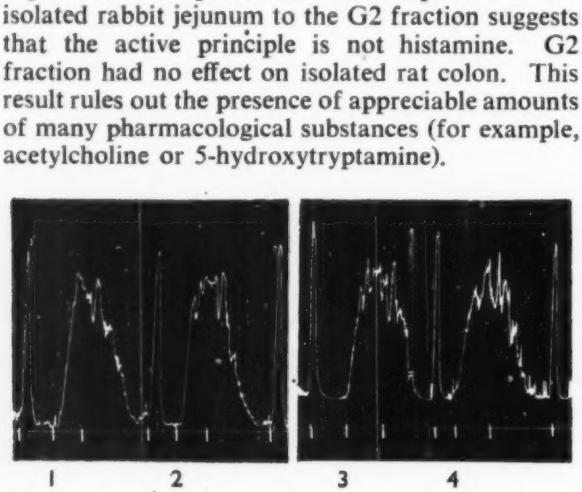

FIG. 2.—The effect of G acid on isolated guinea-pig jejunum. (1) 0.1 mg. G acid. (2) 0.1 mg. G acid. (3) 50 μg. G acid. (4) 50 μg. G acid. Other contractions are due to 0.2 μg. acetylcholine.

Rabbit Coronary Arteries.—0.2 mg. of G acid gave a definite but transitory constriction of the coronary arteries similar to that obtained with 1–2 mg. G2 fraction. The pure substance also produced a moderate and persistent decrease in the amplitude of the heart beat. This also occurred with the G2 fraction, but it was neither

FIG. 3.—Relation between dilution of G acid and rate of haemolysis; 10 ml. phosphate buffer *p*H 7.3; 0.1 ml. 5% erythrocyte suspension.

so definite nor so persistent. These results suggest that some other substance is responsible for most of the constriction of rabbit coronary arteries. The contribution of G acid to the total coronary constrictor activity of the G2 fraction must be very small, for the G acid is 1,000 times more potent than the G2 fraction in producing slow muscular contraction, whereas it is only 7.5 times more potent as a coronary constrictor.

Haemolytic Activity.—Slow muscle-stimulating and coronary-constricting activities are present in

the original G2 fraction, whereas the haemolytic activity of the G acid is completely masked. Fig. 3 shows the relation between dilution of G acid and rate of haemolysis.

Effect of Calcium Ions.—To 5 ml. of 0.1% solution of G acid (sodium salt) in saline, an equivalent amount of calcium chloride in 0.1 ml. water was added. The solution was stirred, allowed to stand at 1° C. for 1 hr., and then filtered. The filtrate was assayed on the isolated guinea-pig ileum and human red cells. The haemolytic activity was completely lost, and the slow contractor activity was reduced by about 90%.

The residual 10% of the slow contractor activity manifested by the so-called pure substance in the presence of an equivalent amount of calcium ions in a protein free system may be attributed to the presence of another substance whose action on the isolated guinea-pig ileum is not affected by calcium ions, or to the possible partial dissociation of the calcium soap formed by G acid at the relatively high dilution of the biological assay. In a 10 ml. Ringer's organ bath where an isolated piece of guinea-pig ileum shows its maximum response to 50 μg. of pure G acid, the amount of calcium chloride present is about 250 times more than the equivalent amount necessary to inhibit 90% of the slow contractor activity of 50 μg. of G acid in a protein free concentrated solution (1 mg. of G acid/ml.). However, calcium ions do not seem to interfere with the slow contractor activity of G acid under the conditions of biological assay. These considerations indicate that the inhibiting effect of calcium ions upon the slow contractor activity of G acid is chemical and not physiological. The same considerations make it obvious that if G acid is present in plasma its slow contractor activity would not be affected by calcium ions, since their concentration in human blood serum (about 5 mg./100 ml. under normal conditions) is less than that present in the Ringer solution used (8.7 mg./100 ml.).

Discussion

Some slow muscle contracting substances seem to arise through the action of proteolytic and lipolytic enzymes on certain precursors. For example, bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin, has been described by Rocha e Silva *et al.* (1949). It has also been reported (Braun-Menéndez *et al.*, 1943) that the action of renin on the pseudo-globulin fraction of normal plasma or serum produces two substances with smooth muscle stimulating actions, one of which is hypertensive (hypertensin) and the other hypotensive.

In 1938 Feldberg and Kellaway, in experiments on perfused lungs, found that the venous perfusate after injection of snake venom caused not only a rapid histamine-like contraction but also a delayed and slow contraction of isolated guinea-pig jejunum. According to these two authors, its formation probably accounted for the stimulating action of the venom on the gut. Also, lymph and egg yolk treated with venom acquired a lytic action on the red blood cells and caused contraction of the isolated jejunum, which had been desensitized to venom. Feldberg and Kellaway thought that these activities were properties of at least two substances, the haemolytic activity being attributed to lysolecithin.

The chemical nature of the slow contractor substance isolated from human plasma suggests that the slow contraction described by Feldberg and Kellaway in their experiments with venom-perfused lungs may have been due to an unsaturated fatty acid identical with, or similar to, G acid. The splitting of this from the parent phospholipid molecule is effected by lecithinase A present in snake venom. When one considers the slow contractor action of G acid in relation to known fatty acids, the following considerations emerge.

Among the saturated fatty acids, those between C_2 and C_{18} were inactive in a dose of 200 μg. The monoethenoid unsaturated C_{18} fatty acids *cis*-9-octadecenoic, *trans*-9-octadecenoic and *cis*-6-octadecenoic acids were also inactive at the same dose. *cis*-11-Octadecenoic acid (*cis*-vaccenic acid), on the other hand, has much the same effect in similar doses as G acid on the isolated guinea-pig jejunum and on rabbit coronary arteries. However, according to the work of Laser (1949) it appears that the haemolytic activity of *cis*-11-octadecenoic acid isolated from horse brain is nearly 3 times that of G acid isolated from the G2 fraction of human plasma. Moreover, a study of the physical and chemical properties of G acid suggests that it differs from *cis*-11-octadecenoic acid with respect to the double bond in the hydrocarbon chain of the fatty acid. The double bond in *cis*-vaccenic acid was proved by Morton and Todd (1950) to be in the 11–12 position. In G acid, however, it seems probable that the double bond occupies the 3–4 position. The diethenoid *cis*-9:12-octadecadienoic acid acts on the isolated guinea-pig jejunum in a dose of 50 μg. The type of contraction and recovery after washing is similar to that after G acid. A dose of 200 μg. of *cis*-9:12-

octadecadienoic acid, however, is practically inactive on rabbit coronaries. In a dose of 200 μg. the triethenoid *cis*-9:12:15-octadecatrienoic acid rendered the isolated guinea-pig jejunum unresponsive to various stimulating agents.

Thus, it may be expected that the slow contractor activity of a given unsaturated fatty acid depends on the number and position of the double bonds in the hydrocarbon chain. These haemolytic and slow contractor activities may be due to different structural areas of the fatty acid molecule. If this is so, then by using such inhibitors of haemolysis as plasma, albumin, or the starting G2 fraction, it should be possible to mask completely the haemolytic activity of the fatty acid in question, and leave the slow contractor activity unaffected. These conditions are fully satisfied with *cis*-9:12-octadecadienoic acid and G acid, which behave similarly.

Summary

1. A slow muscle contractor principle isolated from the G2 fraction of human plasma appears to be a long chain C_{18} unsaturated fatty acid. This principle has been called G acid.

2. It is possible that G acid is 3-octadecenoic acid.

3. G acid manifests the following three biological properties: (a) A slow and delayed contraction of the isolated jejunum of the guinea-pig in a concentration of 2×10^{-6}; (b) a marked haemolytic activity on human red cells in a concentration of 2×10^{-6}; and (c) a vasoconstrictor activity on rabbit coronary arteries in a dose of 0.2 mg. in the isolated perfused heart.

4. It seems probable that the centre of unsaturation in G acid is responsible for its biological properties.

5. The probable relation of G acid to the slow acting principles obtained by previous workers and to known fatty acids is discussed.

My thanks are due to Sir Alan Drury, F.R.S., for his help and advice.

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SLOW HYPERFIBRINOGENAEMIC ACTION OF ADRENALINE AND RELATED SUBSTANCES

BY

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(RECEIVED JULY 1, 1955)

A single subcutaneous injection of adrenaline produces a rise in the concentration of plasma fibrinogen in the rat (Henriques, Henriques, and Mattos, 1950). This rise is proportional to the logarithm of the dose in the range 12.5–200 μg./100 g. body weight. A dose of 50 or 100 μg. adrenaline, given subcutaneously or intraperitoneally, caused a marked increase of fibrinogen 24 hr. later, but no change was detected 1, 2, or 4 hr. after administration. This lag between injection and response seems to distinguish the phenomenon—which had also been reported briefly by Henriques, Henriques, and Selye (1950)—from similar results recorded in the earlier literature. Thus, Riecker and Winters (1931), Goreczky and Kovats (1942), and Sütö-Nagy (1944) reported a transitory rise of plasma fibrinogen a few minutes after the administration of adrenaline to dogs or men. Biggs, MacFarlane, and Pilling (1947) have reported a transient increase in fibrinolysis after adrenaline administration to human subjects.

This paper describes experiments on the time-relation between treatment and appearance of fibrinogen increase, on the relation between chemical structure and hyperfibrinogenaemic action of sympathomimetic amines, and on the blocking by tolazoline ("Priscol") of the hyperfibrinogenaemic effect of adrenaline.

MATERIAL AND METHODS

The adrenaline used was either (±)-adrenaline hydrochloride (Sterling-Winthrop), or a 1:1,000 solution of "Adrenalin" (Parke, Davis & Co.). The latter was shown by chromatographic analysis to contain a small proportion of (−)-noradrenaline; Auerbach and Angell (1949) found from 10.5 to 18.5% of (−)-noradrenaline in different samples of U.S.P. adrenaline. The (±)-noradrenaline and *N-iso*propylnoradrenaline hydrochlorides used were also obtained from Sterling-Winthrop. Amphetamine hydrochloride was kindly supplied by Dr. M. Rocha e Silva. Other drugs used were histamine dihydrochloride (Paul-Lewis Laboratories); phenylephrine (Neosynephrine, Winthrop Products); pholedrine (Veritol, Knoll A.G.); ephedrine (British Drug Houses); and tolazoline (Priscol, Ciba). The hydrochlorides of tyramine, hydroxytyramine, *N*-methylhydroxytyramine, noradrenalone, *N*-butylnoradrenalone, *N*-methyladrenalone were synthesized in our laboratory by standard techniques. The purity of these substances was verified by melting point determinations and by chromatography on Whatman No. 1 filter paper, using butanol, acetic acid and water as solvent and $FeCl_3$-K_4[$Fe(CN)_6$] as developer (Barton, Evans, and Gardner, 1952). All substances gave only one spot on the chromatograms, except *N*-methylhydroxytyramine which contained traces of hydroxytyramine. Sometimes the purity was also checked by nitrogen determination. (−)-Adrenochrome was prepared by the method of Sobotka and Austin (1951); no adrenaline was detectable in it by the toad heart test.

Experimental Techniques.—Albino rats, 100–150 g. body weight, were used. To test the hyperfibrinogenaemic action, a substance was injected subcutaneously, the controls receiving an equal volume of 0.9% NaCl, and the animals were bled and killed on the next day. In the experiments done to determine the blocking activity of tolazoline on lung oedema produced by "Adrenalin," tolazoline was given subcutaneously 30 min. before the "Adrenalin" was injected intravenously through a tail vein. When testing the influence of tolazoline on the hyperfibrinogenaemic action of "Adrenalin," tolazoline was given 30 min. before "Adrenalin"; both drugs were given by subcutaneous injection. In a few experiments the treatment with tolazoline was repeated before the "Adrenalin" injection. The animals were always bled through the aorta, under pentobarbitone (40 mg./kg.) or ether anaesthesia, by means of a dry syringe coated with Dry Film 9987, according to the technique of Jaques, Fidlar, Feldsted, and MacDonald (1946). The blood was immediately transferred to a tube containing 0.01 ml. of 30% potassium oxalate/ml. blood and centrifuged. In a few experiments part of the blood was allowed to clot, the serum being used for the determination of total and non-protein nitrogen.

Analytical Methods.—For fibrinogen determinations, the fibrin, isolated from 1 ml. of blood plasma by the method of Cullen and Van Slyke (1920), was transferred to a Kjeldahl micro-flask and ashed by the method of Campbell and Hanna (1937). The ammonia thus formed was distilled and collected in excess standard acid, which was subsequently titrated. Finally ammonia nitrogen was converted into fibrinogen nitrogen using 6.00 as conversion factor (Morrison, 1947). By means of the same ashing and titration technique total nitrogen was determined in 0.1 ml. of blood serum, and non-protein nitrogen (NPN) was determined in 5 ml. of Folin-Wu blood serum filtrate (Folin and Wu, 1919). Protein nitrogen, obtained by difference between total nitrogen and NPN, was converted into the total serum protein by using 6.25 as conversion factor.

Results

Influence of Time on the Hyperfibrinogenaemic Action of "Adrenalin."—Table I shows that "Adrenalin," 200 μg./100 g. body weight subcutaneously, caused a significant rise of fibrinogen as soon as 4 hr. after injection. After a single injection the fibrinogen concentration was still increasing between 8 and 12 hr. after treatment.

TABLE I

INFLUENCE OF TIME ON THE HYPERFIBRINOGENAEMIC ACTION PRODUCED IN THE RAT BY SUBCUTANEOUS ADMINISTRATION OF "ADRENALIN" (PARKE, DAVIS & CO.)

Dose (μg./100 g.)	Time After Injection (hr.)	No. of Animals	Plasma Fibrinogen (mg./100ml. ± S.E.)
200	4	9	318 ± 17·7
200	8	7	362 ± 9·5
200	12	9	507 ± 22·8
400*	24	6	699 ± 19·6
Saline control	24	10	255 ± 13·8

* Given in two doses, interval 3 hr., and the rats bled 24 hr. after the first injection.

Relation between Chemical Constitution and the Hyperfibrinogenaemic Action of Sympathomimetic Amines.—The following amines caused a significant rise in the concentration of blood plasma fibrinogen: Adrenaline was the most active; *N-iso*propylnoradrenaline, noradrenaline and phenylephrine were intermediate, and *N*-methylhydroxytyramine, adrenalone, hydroxytyramine and pholedrine were much less active (Table II). All the other compounds, including adrenochrome, proved to be inactive in the dose administered.

The relative potencies shown in Table II were calculated by dividing the dose of adrenaline, which should produce the same effect as the substance studied ("adrenaline equivalent"), by the dose of the sympathomimetic amine used in the experiment. The "adrenaline equivalent" is the antilog of $\left[\frac{F_U}{F_A}\log A\right]$, where F_U is the difference in blood fibrinogen concentration between the average of the groups treated with the amine of unknown activity and the average of the control group; F_A is the similar difference between adrenaline-treated and control groups; A is the adrenaline dose used in the corresponding experiment. Nevertheless this calculation can only give an approximation to the true relative potencies of the different substances. The design of the experiment would not allow an appraisal of the errors involved, and therefore differences as small as the one observed between (±)-noradrenaline and

TABLE II

HYPERFIBRINOGENAEMIC ACTION OF SYMPATHOMIMETIC AMINES

The potencies are related to that of (±)-adrenaline taken as 1 (see Results for method of calculation). All the rats were bled 24 hr. after a single subcutaneous injection of the amine.

Expt.	Substance	Dose (mg./100g.)	No. of Animals	Fibrinogen (mg./100 ml. ± S.E.)	*P* Related to Saline Control	Relative Potency
I	(±)-Noradrenaline	0·1	20	269 ± 10·7	0·002	0·04
	(±)-Adrenaline	0·1	21	361 ± 9·7	0·001	1
	(±)-*N-iso*propylnoradrenaline	0·1	20	335 ± 9·2	0·001	0·40
	Saline control	—	21	228 ± 6·6	—	
II	Phenylephrine	1	11	321 ± 14·9	0·001	0·03
	(±)-Adrenalinecontrol	0·1	12	353 ± 21·0	0·001	1
	Saline control	—	12	233 ± 7·2	—	
III	Hydroxytyramine	2	10	245 ± 13·7	0·007	0·001
	N-Methylhydroxytyramine	2	9	266 ± 16·1	0·001	0·002
	Tyramine	2	9	219 ± 6·9	0·048	0·001
	(±)-Adrenalinecontrol	0·2	9	507 ± 34·4	0·001	1
	Saline control	—	10	199 ± 6·8	—	
IV	Adrenalone	2	10	268 ± 10·0	0·008	0·002
	Noradrenalone	2	10	246 ± 12·3	0·19	0·001
	N-Butylnoradrenalone	2	9	223 ± 10·9	0·90	0
	N-Methyladrenalone	2	10	241 ± 10·6	0·24	0·001
	(±)-Adrenalinecontrol	0·2	10	430 ± 20·5	0·001	1
	Saline control	—	10	221 ± 12·3	—	
V	Amphetamine	1	10	223 ± 18·4	0·49	0·001
	Ephedrine	1	9	236 ± 17·2	0·14	0·002
	Pholedrine	1	10	253 ± 14·4	0·014	0·003
	(±)-Adrenalinecontrol	0·1	10	368 ± 17·8	0·001	1
	Saline control	—	10	209 ± 7·3	—	
VI	(−)-Adrenochrome	0·4	10	254 ± 14·0	0·23	0·005
	,, ,,	0·8	10	254 ± 16·0	0·27	0·002
	(±)-Adrenalinecontrol	0·2	10	406 ± 12·5	0·001	1
	Saline control	—	9	230 ± 15·8	—	

TABLE III

INFLUENCE OF ADRENALINE ON THE CONCENTRATION OF PLASMA FIBRINOGEN, SERUM PROTEINS AND NON-PROTEIN NITROGEN

Adrenaline and saline were given in two subcutaneous injections, interval 5 hr., and the rats bled 24 hr. after the first injection. Number of rats used is indicated in parentheses

Expt. No.	(±)-Adrenaline (μg./100 g.)	Plasma Fibrinogen		Serum Proteins		Non-protein Nitrogen	
		mg./100 ml. ±S.E.	*P*	g./100 ml. ±S.E.	*P*	mg./100 ml. ±S.E.	*P*
I	180	484±13·7 (10)	0·001	6·06±0·01 (8)	0·27	31·9±1·27 (8)	0·007
	Saline control ..	196± 6·2 (10)		5·78±0·18 (9)		27·2±0·86 (9)	
II	200	488±29·2 (10)	0·001	6·24±0·23 (10)	0·10	—	—
	Saline control ..	189± 5·81 (10)		5·81±0·06 (10)			

phenylephrine (0.03 and 0.04) may not indicate a real difference in activity. The figures are therefore only rough estimates of the hyperfibrinogenaemic activity of the amines.

Influence of Adrenaline on the Plasma Fibrinogen, Serum Proteins and Non-protein Nitrogen (NPN) Concentration.—Adrenaline more than doubled the concentration of fibrinogen, producing at the same time a significant increase in the NPN concentration (Table III). In two experiments the total serum protein was higher in adrenaline-treated than in control animals, but this difference was not statistically significant.

Action of Other Nitrogenous Substances on the Concentration of Plasma Fibrinogen.—Tetraethylammonium and tolazoline did not affect plasma fibrinogen in a dose of 1 to 1.25 mg./100 g. body weight, whereas histamine increased it in a dose of 6.5 mg./100 g. but not when the dose was 0.65 mg./100 g. or less (Table IV).

Influence of Tolazoline on the Hyperfibrinogenaemic Activity of "Adrenalin."—In order to estimate the effective dose range of tolazoline in the rat, we measured its ability to prevent prompt death caused by the intravenous injection of "Adrenalin." Examination of the animals which died after intravenous injection of "Adrenalin" showed that they had lung oedema, since all of them presented a flow of foamy haemorrhagic fluid coming out of their noses. Preliminary experiments gave an LD50 for "Adrenalin" of 21 μg./100 g. (95% confidence limits 19.2–24.4 μg.). Table V shows the relation between the doses of tolazoline and the incidence of lung oedema in rats treated intravenously with 66 μg./100 g. of "Adrenalin." The mortality rate was 50% after treatment with 1.25 mg. tolazoline (95% confidence limits of 1.22 to 1.28). Therefore 1.25 mg. tolazoline seemed to have abolished the effect of 45 μg. "Adrenalin."

TABLE IV

PLASMA FIBRINOGEN CONCENTRATION OF RATS 24 HOURS AFTER THE SUBCUTANEOUS ADMINISTRATION OF SEVERAL NITROGENOUS SUBSTANCES

All the rats were bled 24 hr. after a single subcutaneous injection of the substance

Expt. No.	Substance	Dose (mg./150 g.)	No. of Animals	Fibrinogen (mg./100 ml. ±S.E.)
I	Tetraethylammonium	2	6	195± 3·17
	Tolazoline ..	2	6	202± 10·0
	"Adrenalin," P.D.	0·1	6	308±18·5
II	Histamine ..	0·02	11	182± 6·91
	"	0·10	10	190± 7·76
	"	1	11	211± 7·88
	"	10	8	274± 7·11
	Saline control ..	—	8	218± 7·69

TABLE V

INHIBITION BY TOLAZOLINE OF THE LETHAL EFFECT OF INTRAVENOUS ADMINISTRATION OF 66 μG. "ADRENALIN" (PARKE, DAVIS) PER 100 G. OF BODY WEIGHT IN RATS

Tolazoline given s.c. 30 min. before "Adrenalin"

Dose of Tolazoline (mg./100 g.)	No. of Rats	Mortality %
0·83	10	80
1·00	19	63
1·17	10	50
1·42	10	60
1·67	10	30
2·00	10	10
4·00	10	0

The effect of pre-treatment with tolazoline on the hyperfibrinogenaemic action of "Adrenalin" was studied in several experiments. Judging from the ability of tolazoline to prevent death from "Adrenalin," the dose of tolazoline used should have been enough to inhibit the effect of the "Adrenalin" administered in all experiments except No. 1. The results (Table VI) show that in all experiments the average fibrinogen concentration of the tolazoline-"Adrenalin"-treated rats was smaller than that of the saline-"Adrenalin"-treated ones, although the difference was significant only in Expt. II and in one group of Expt. IV.

TABLE VI

INFLUENCE OF TOLAZOLINE ON THE SLOW HYPERFIBRINOGENAEMIC ACTION PRODUCED BY SUBCUTANEOUS ADMINISTRATION OF "ADRENALIN" IN THE RAT

The first tolazoline injection was given subcutaneously 30 min. before "Adrenalin" (Parke, Davis)

Expt. No.	No. of Rats	Dose (mg./150 g.) of		Fibrinogen (mg./100 ml. ±S.E.)
		Tolazoline (mg.)	"Adrenalin" (mg.)	
I	6	2	0·1	337±21·6
	7	—	0·1	382±27·9
II	10	3×2*	0·1	290±11·0
	10	3×2*	—	250±12·2
	10	—	0·1	347±12·0
III	7	4+2**	0·2***	315±15·0
	9	4+2**	—	272±17·0
	8	—	0·2	363±35·0
IV	8	4+2**	0·2	374±16·8
	7	4+2**	0·2	377±22·9
	8	6	0·2	376±14·9
	7	—	0·2	434±22·7
	8	4+2**	—	350±19·2

* Given in 3 doses, interval 2 hr. ** Given in 2 doses, interval 2 hr. *** "Adrenalin" was given mixed together with the first tolazoline injection. Sum of $\chi^2_8 = 22{\cdot}402$; $P = 0{\cdot}01$ for comparison between saline-"Adrenalin"- and tolazoline-"Adrenalin"-treated rats. Sum of $\chi^2_6 = 19{\cdot}018$; $P = 0{\cdot}002$ for the comparison between tolazoline-"Adrenalin"- and tolazoline-saline-treated rats.

On the other hand, the tolazoline-"Adrenalin"-treated rats had higher fibrinogen concentration than the tolazoline-saline-treated ones, but only in one experiment was the difference statistically significant. A χ^2 analysis of the results (Fisher, 1944) showed that tolazoline inhibits the hyperfibrinogenaemic action of "Adrenalin" ($\chi^2_8 = 22.402$; $P = 0.01$), but this inhibition is not complete ($\chi^2_6 = 19.018$; $P = 0.002$), with the dose administered in these experiments.

DISCUSSION

From our results, the hyperfibrinogenaemic action of sympathomimetic amines seems to be a consequence of very specific process. Thus, of the substances studied, adrenaline seemed to have the most suitable structure for hyperfibrinogenaemic action. Removal of one of its phenolic groups (phenylephrine) or of its *N*-methyl group (noradrenaline) decreased the potency to 1/20 to 1/30, and reduction of its alcoholic group to methylene (*N*-methyl-hydroxytyramine) or oxidation of the same group to ketone (adrenalone) reduced the activity to about 1/500. A substituent in the amine group improves the activity, for *N-iso*propylnoradrenaline, although less active than adrenaline, was more active than noradrenaline. This improvement in activity in the *N*-substituted derivatives of noradrenaline is a well-known feature of other pharmacological actions of adrenaline homologues, such as the inhibitory action on smooth muscle (Barger and Dale, 1910; Marsh, Pelletier, and Ross, 1948; Bacq and Lecomte, 1949; Bacq, Fischer, and Lecomte, 1949; Lands, 1949), on glycogenolysis (McChesney, McAuliff, and Blumberg, 1949; Ellis, 1951), and on the adrenaline-induced decrease in the ascorbic acid content of the adrenal glands (Jarrett, 1951). Of these, the activity which seems to be more closely related to the hyperfibrinogenaemic effect is the glycogenolytic action which can take place in the liver, where most of the fibrinogen is known to be formed (Miller, Bly, Watson, and Bale, 1951). McChesney *et al.* (1949) and Ellis (1951) observed that adrenaline possesses maximal hyperglycaemic activity, that noradrenaline has a potency of about 1/15 of this, and that *N-iso*propylnoradrenaline has very little activity. Therefore one could assume that, if both hyperfibrinogenaemic and hyperglycaemic activities take place in the same cells, the receptors involved should have different structural specificities; but for this assumption to be justified it would be necessary to show that the hyperfibrinogenaemic effect is caused by a direct action of adrenaline on the liver cells, as seems to be so far the hyperglycaemic effect (Cross and Holmes, 1937; Bendall and Lehmann, 1941). Tolazoline, however, seems to be able to block both the hyperfibrinogenaemic activity (present paper) and the hyperglycaemic activity of adrenaline (Ellis and Anderson, 1950; and Harvey, Wang, and Nickerson, 1952).

We found no change in the concentration of blood plasma fibrinogen in rats receiving 15 μg. "Adrenalin"/100 g. body weight, given into a tail vein. This result contrasts with that published by Henriques *et al.* (1950) showing an increase in fibrinogen after the subcutaneous administration of 12.5 μg./100 g. body weight, a discrepancy which might be explained by the very slow absorption of subcutaneous adrenaline (Cori and Cori, 1930a; Schayer, 1951). This, however, would not exclude the possibility of a direct effect on the liver cells, since the amine might work rather slowly; a better experiment would involve a prolonged intravenous infusion as done by Cori and Cori (1930b) to study the mechanism of adrenaline hyperglycaemia. Another possible explanation for adrenaline-induced hyperfibrinogenaemia would be the liberation of some active agent, set free by tissue damage caused by adrenaline ischaemia at the site of its administration, since it is well known that tissue damage causes an increase in the concentration of blood plasma fibrinogen (Foster and Whipple, 1922; Chanutin, Hortenstine, Cole, and Ludewig, 1938). Against this

interpretation we have the fact that in the rat, in the doses used by us, adrenaline does not produce any visible tissue damage. It is also noteworthy that *N-iso*propylnoradrenaline, which is considered a vasodilator agent (Konzett, 1940; Marsh *et al.*, 1948; Ahlquist, 1948), is the second most potent hyperfibrinogenaemic substance. In contrast, noradrenaline, which is generally admitted to have only vasoconstrictor activity, has a much lower fibrinogen-increasing effect. The conclusion as to the mechanism of adrenaline hyperfibrinogenaemia, whether by a direct action on the fibrinogen producing cells or by means of a mediator, must wait for more direct evidence.

The results presented suggest that the adrenaline-induced increase of fibrinogen is the consequence of a slow and very long process, since the concentration of fibrinogen was still rising after 12 hr. This might be explained by assuming that the normal metabolites of adrenaline are also active. Against this idea, adrenochrome, which has been considered an adrenaline metabolite (Bacq, 1949) and has some of the pharmacological actions of adrenaline (Bacq, 1947), did not produce any fibrinogen increase even in a dose of 800 μg. This, however, does not exclude the possibility of other metabolites being active, and indeed Schayer and Smiley (1953) have presented strong evidence that adrenochrome is not an intermediate in adrenaline metabolism.

In the experiments presented in this paper we have also found that the rats treated with adrenaline had a higher NPN concentration than the control animals. This confirms previous findings (Watkins and Smith, 1931).

Summary

1. A series of sympathomimetic amines and related substances were injected subcutaneously into rats. The following caused a significant rise in the concentration of blood plasma fibrinogen 24 hr. later: "Adrenalin," Parke, Davis & Co., (±)-adrenaline, (±)-*N-iso*propylnoradrenaline, (±)-noradrenaline, phenylephrine, *N*-methyl-hydroxytyramine, adrenalone, hydroxytyramine, and pholedrine. Adrenaline was the most potent, (±)-*N-iso*propylnoradrenaline had about 40%, and (±)-noradrenaline and phenylephrine had about 3%, of the activity of adrenaline.

2. Tolazoline (4 mg./100 g. body weight) partially abolished the hyperfibrinogenaemic effect of 100–200 μg. adrenaline.

Our thanks are due to Drs. M. Rocha e Silva and Ulla Hamberg for helpful criticism and to Dr. S. Schenberg for some pharmacological tests on the toad heart. The work was done during the tenure by one of us (A. L.) of a Brazilian Research Council Fellowship.

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Brit. J. Pharmacol. (1956), **11**, 104.

A SIMPLE NEW QUANTITATIVE METHOD FOR TESTING LOCAL ANAESTHETICS

BY

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(RECEIVED AUGUST 19, 1955)

Local anaesthetic activity is usually determined either by the method originally described by Regnier (1923) and later modified by other authors (inhibition of the corneal reflex by local application of drug), or by the method of Bülbring and Wajda (1945) (inhibition of the reaction to pin-prick stimulus of the skin by intracutaneous injection of drug).

The first method measures surface anaesthetic activity ; the second method also tests the power of infiltration. The power of the local anaesthetic to block nerve conduction is much less frequently determined. Either the method of Bülbring and Wajda (1945) (inhibition in the decapitated eviscerated frog of reflex contraction of the leg after stimulation by immersion in HCl, after injection near the plexus ischiaticus), Shackell's method (1935) (inhibition in the guinea-pig of the pain reflex provoked by stimulation of the foot after injection near the nervus ischiaticus), or the method of Dietrichs (1931) (inhibition of the reflex contraction to electric stimulation of the ischiaticus gastrocnemius preparation of the frog with local application of the drug to the nerve), may be used.

Bianchi (unpublished) measures local anaesthetic activity by the method reported by Kisch (1948), but using *Rana temporaria* instead of the bullfrog. The frogs lose their righting reflex for a certain period of time after intracranial injection of the drug. Unfortunately, however, this method is not specific, since hypnotics such as phenobarbitone produce the effect as well as local anaesthetics.

Mack and Nelson (1953), and Herr, Nyiri, and Pataky (1953), applied to local anaesthetics the radiant heat method of Hardy, Wolff and Goodell (1940) for the determination of analgesic activity. The time required for the reappearance of the pain reflex in the rat's tail is measured after subcutaneous injection of the compound.

In order to overcome some difficulties encountered during the application of the above methods in screening new compounds with supposed local anaesthetic activity, we thought it desirable to try Haffner's method (1929) (application of pressure on the mouse tail's root), which, as demonstrated by Bianchi and Franceschini (1954), is suitable for the quantitative estimation of analgesic drugs.

METHOD

Fully grown albino mice of either sex may be used. A small artery clip with its blades covered by thin rubber tube is applied to the root of the tail. Those animals which do not show within 30 sec. the pain reflex (the mice turn again and again trying to remove the clip) are eliminated. The remainder receive subcutaneously, about 1 cm. from the root of the tail, 0.1 ml. of a solution of the drug. 15 min. after injection the pain reflex of all the injected animals is tested, applying the stimulus to the zone where the compound was injected.

The proportion of animals which proved to be anaesthetized (animals which within 30 sec. after the application of the clip do not show the usual pain reflex) was noted for each dose.

The following drugs were tested in 2% (w/v) solution : cocaine hydrochloride, procaine hydrochloride, lidocaine (lignocaine) hydrochloride, and cinchocaine hydrochloride.

These solutions were further diluted with saline to obtain the concentration desired.

To all solutions adrenaline hydrochloride (10 μg./ml.) was added.

RESULTS

Injections of saline solutions with adrenaline do not affect the pain reflex.

There is a linear relation between the log of the concentration of local anaesthetic and the probit of the percentage of mice showing anaesthesia (Fig. 1). The slopes of the dose-response lines obtained with different local anaesthetics may be regarded as parallel ($P=0.05$). The ED50, and S, with fiducial limits, have been calculated graphically for each drug by the method of Litchfield and Wilcoxon (1949). The results obtained are shown

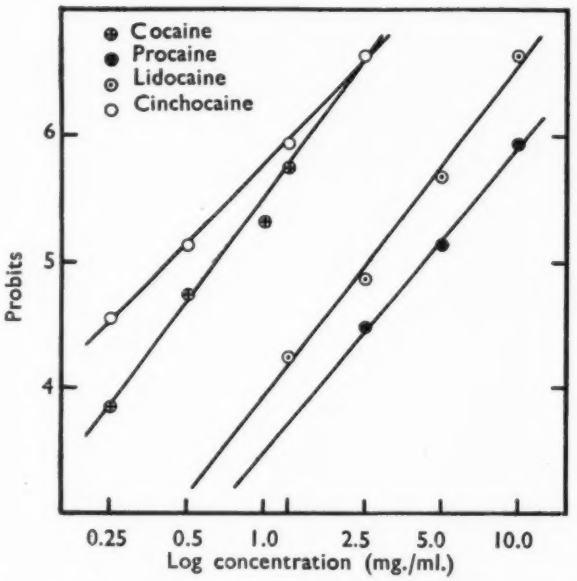

FIG. 1.—Showing the anaesthetic activity of various drugs when tested 15 min. after subcutaneous injection to tails of mice. Abscissa, log concentration in mg./ml. Ordinate, probits.

in Table I. The ED50 of cocaine in this series was 0.66 mg./ml. In another group of 75 mice the ED50 of cocaine was 0.70 mg./ml. (with fiducial limits of 0.58–0.84 mg./ml.), which agrees well with the first result. The activity of commercial preparations of procaine was tested and the results showed no significant difference from those of pure procaine HCl (Table II).

The anaesthetic activity of all these compounds is of short duration and wears off within 90 min.

The activity of cocaine is nearly half that of cinchocaine, approximately four times that of lidocaine, and seven times that of procaine. The activity of lidocaine is about twice that of procaine.

TABLE I

LOCAL ANAESTHETIC ACTIVITY TESTED 15 MIN. AFTER SUBCUTANEOUS INJECTION OF THE DRUGS TO TAILS OF MICE

(The numerals in parentheses are the 19/20 fiducial limits)

Drug	No. of Mice	ED50 (mg./ml.)	S	Activity Ratios (Cocaine = 1)
Cocaine HCl ..	160	0·66 (0·53–0·81)	2·31 (1·67–3·18)	1
Procaine HCl ..	120	4·25 (3·40–5·31)	2·56 (1·69–3·86)	0·15 (0·10–0·20)
Lidocaine HCl	160	2·60 (2·00–3·20)	2·40 (1·81–3·16)	0·25 (0·18–0·34)
Cinchocaine HCl	140	0·42 (0·31–0·55)	3·02 (2·01–4·53)	1·57 (1·20–2·29)

DISCUSSION

The potency ratios of local anaesthetics obtained with this new method agree with those obtained by some other methods (Goodman and Gilman, 1955; Krantz and Carr, 1954), but not always with those reported by other authors,—for example, Mongar (1955). It may be that differences in the technique used account for the discrepancies in the activity ratios found. Indeed, the presence of buffer solutions such as phosphate increases the activity of the local anaesthetics. Mongar showed that, when the drugs are administered in buffered solutions, cinchocaine is 34 times more active than procaine by the guinea-pig weal method. Elio (1948), with the same technique but using unbuffered solutions, found cinchocaine to be only ten times more active than procaine.

TABLE II

LOCAL ANAESTHETIC ACTIVITY TESTED 15 MIN. AFTER SUBCUTANEOUS INJECTION OF COMMERCIAL SAMPLES OF PROCAINE TO TAILS OF MICE

(The numerals in parentheses are the 19/20 fiducial limits)

Sample	No. of Mice	ED50 (mg./ml.)	S	Activity Ratios (Procaine = 1)
Procaine HCl ..	120	4·25 (3·40–5·31)	2·56 (1·69–3·86)	1
1	180	5·80 (4·00–8·40)	6·20 (2·60–14·2)	0·73 (0·47–1·12)
2	160	5·90 (4·70–7·40)	2·14 (1·62–2·76)	0·72 (0·53–0·97)
3	140	4·40 (3·57–5·41)	1·87 (1·55–2·24)	0·96 (0·71–1·29)
4	60	5·00 (3·33–7·50)	3·21 (1·14–8·98)	0·85 (0·53–1·35)

The technique described in this paper offers the advantages of easy and rapid performance, and of results which are both quantitatively and readily compared. The performance of the test does not require any particular ability; even the quantity of solution to be injected does not have to be kept absolutely constant. Indeed, there is frequently leakage from the site of injection owing to the high local pressure. During the great number of tests performed, only once have we encountered a dose response line with a slope less than that usually found: the reason for this was not apparent.

SUMMARY

1. A simple new method for testing local anaesthetic properties of drugs on mice is described.

2. The activities of cocaine, procaine, lidocaine, and cinchocaine were compared; the results agree with those quoted by other authors.

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Brit. J. Pharmacol. (1956), **11**, 107.

ACTIVATION OF RABBIT SERUM PROTEASE BY DEXTRAN SULPHATE

BY

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(RECEIVED SEPTEMBER 1, 1955)

Evidence that activation of a proteolytic enzyme may play a part in anaphylactic reactions has been furnished by Rocha e Silva (1950) and Ungar and his co-workers (Ungar, 1947; Ungar and Mist, 1949). Their results are in accord with the hypothesis put forward by Bronfenbrenner (1948), in which histamine release is visualized as taking place during anaphylactic reactions as a consequence of proteolysis.

Among substances which produce anaphylactoid reactions, both peptone (Scroggie, Jaques, and Rocha e Silva, 1947) and trypsin (Kocholaty, Ellis, and Jensen, 1952; Dragstedt and Rocha e Silva, 1941) release cellular histamine and activate serum protease. Various polysaccharides activate protease when incubated with serum or plasma from certain species (Ungar, 1947; Geiger, 1952), and dextran sulphates which give rise to anaphylactoid reactions in guinea-pigs (Walton and Ricketts, 1954) release cellular histamine (Haining, 1955). The present work shows that certain fractions of dextran sulphate are also capable of activating serum protease.

METHODS

Blood was obtained from rabbits as described previously (Haining, 1955). When plasma was required, sodium oxalate at a final concentration of 1 mg./ml. was used instead of heparin. A volume of 1.5 ml. of serum or dilute plasma was incubated with either 0.5 ml. of a solution of 0.9% (w/v) NaCl, or 0.5 ml. of the same solution plus activator, for 3 min. at 37.5° C. and was then diluted with 40 ml. of distilled water. The euglobin fraction was precipitated and the proteolytic activity was estimated by the method described by Geiger (1952). Increases in optical density, due to colour-producing breakdown products of digested bovine fibrinogen, were converted to μg. of L-tyrosine by reference to a standard curve.

The code numbers of the dextran and dextran sulphate fractions used and their physical and biological properties have been described previously (Ricketts, 1952a, b; Ricketts and Walton, 1952; Walton, 1952; Haining, 1955).

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RESULTS

Sodium oxalate prevents the *in vitro* release of histamine from rabbit blood cells by dextran sulphate (Haining, 1955). Since this might be due to inhibition of an enzyme, oxalate plasma seemed unsuitable for tests of protease activation. Heparinized plasma was also excluded because low concentrations of heparin activate protease, whereas higher concentrations inhibit activation (Ungar and Mist, 1949). Investigations were therefore begun with fresh rabbit serum.

When rabbit serum was incubated with dextran sulphate D/3 for 3 min. and the precipitated euglobin fraction incubated with denatured bovine fibrinogen, the amounts of colour-forming breakdown products increased with increasing concentrations of dextran sulphate from 4 to 100 μg./ml. When the concentration was increased to 500 μg./ml. there was a large reduction in protease activity (Table I).

TABLE I

ACTIVATION OF PROTEASE IN RABBIT SERUM BY DEXTRAN SULPHATE D/3

Conc. of Dextran Sulphate D/3 (μg./ml.)	Protease Activity (L-Tyrosine Equivalent, μg./ml. of Serum)						P
	Rabbit 27	Rabbit 33	Rabbit 26	Rabbit 32	Rabbit 38	Mean	
Control	14	16	6	28	33	19·4	—
4	30	30	20	18	48	29·2	>0·05
20	39	23	25	51	77	43·0	<0·02
100	63	72	46	74	86	68·2	<0·01
500	1	−6	29	8	0	6·4	>0·05

These results were strikingly similar to the effect of incubating samples of diluted rabbit blood with varying concentrations of dextran sulphate D/3, and to the resulting levels of plasma histamine (Fig. 1). The range of concentrations over which dextran

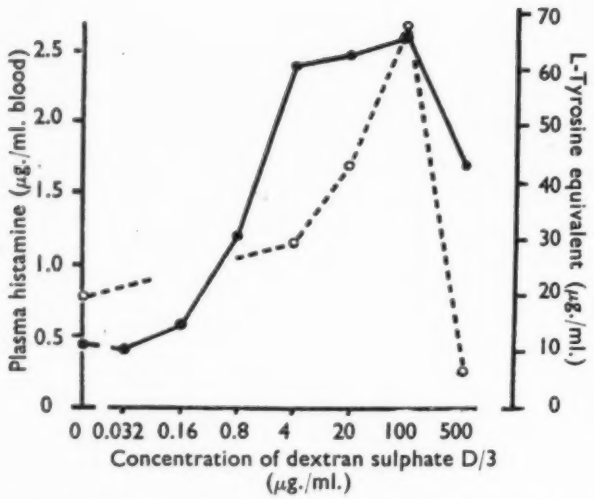
Plasma histamine (μg./ml. blood)
L-Tyrosine equivalent (μg./ml.)
0 0.032 0.16 0.8 4 20 100 500
Concentration of dextran sulphate D/3 (μg./ml.)

FIG. 1.—The release of histamine from rabbit blood cells and activation of protease in rabbit serum by dextran sulphate D/3. Closed circles, plasma histamine levels after incubating dilute heparinized rabbit blood with dextran sulphate D/3 for 30 min. at 37·5° C. Each point represents the mean value obtained with blood from the same 3 rabbits. Open circles, protease activity in serum after incubation with dextran sulphate D/3 for 3 min. at 37·5° C. Each point represents the mean value obtained with serum from the same 5 rabbits.

sulphate D/3 gave rise to protease activation in serum was within that previously found to release histamine in blood. A concentration of 500 μg./ml. inhibited both histamine release and protease activation.

The effect of incubating a mixture of euglobin fraction activated by dextran sulphate D/3 with a solution of denatured bovine fibrinogen for varying periods of time is shown in Fig. 2. Heating to 80° C. for 15 min. was sufficient to inactivate the euglobin fraction (Fig. 3). Dextran sulphate D/3 did not cause proteolysis when incubated with denatured bovine fibrinogen for 2 hr. at a concentration of 33 μg./ml., the concentration which would have been present if it were all precipitated with the euglobin fraction in the experiments of Table I.

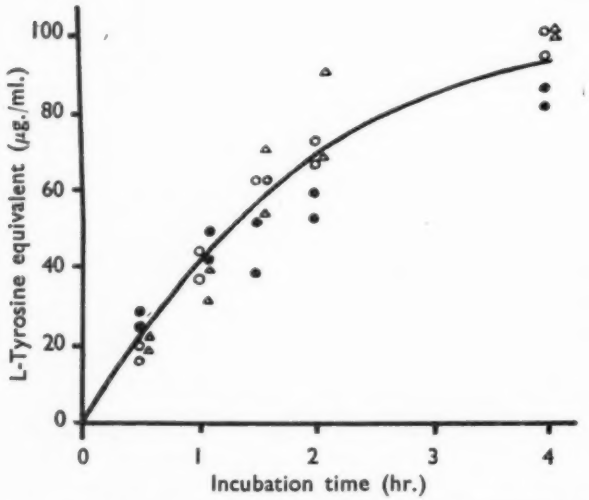

FIG. 2.—Digestion of heat-denatured bovine fibrinogen by the protease of rabbit serum. The protease was activated by incubating serum with dextran sulphate D/3 (100 μg./ml.) for 3 min. at 37·5° C.

The mean molecular weight of a fraction of dextran sulphate has been shown to be a factor in determining its ability to release histamine from rabbit blood cells *in vitro*. If protease activation were concerned in the histamine release reaction, those samples capable of releasing histamine would be expected to activate the protease in rabbit serum also. Three fractions of dextran sulphate were tested, D/3, E/1, and I; the corresponding molecular weights were approximately 440,000, 40,000, and less than 10,000. These were employed at a concentration of 100 μg./ml., since previous experiments had shown that this gave maximum activation in rabbit serum using dextran sulphate D/3. Significant increases in proteolysis were observed with dextran sulphate D/3 or E/1 ($P < 0.05$) but not with fraction I (Table II*a*).

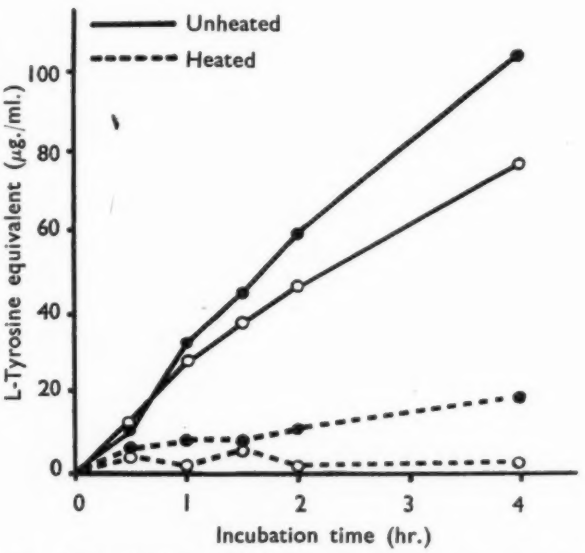

FIG. 3.—Inactivation of rabbit serum protease by heat. Open and closed circles distinguish the 2 different samples of serum used. Continuous lines join values obtained with unheated serum and dotted lines those obtained with serum which had been heated at 80° C. for 15 min. and then rapidly cooled.

When dextran sulphate D/3 was incubated with guinea-pig or pooled rat serum, the results were similar to those obtained with rabbit serum. At a concentration of 100 μg./ml. there was activation of serum protease, but with a higher concentration no

significant increase was obtained. With rat serum 500 μg./ml. gave a value very close to that of the control, and in guinea-pig serum a concentration of 700 μg./ml. had a similar effect (Table II*e* and *g*). Dextran sulphate D/3 also activated protease in oxalated rabbit plasma; concentrations of 4, 20, and 100 μg./ml. of diluted plasma caused graded increases in proteolysis (Table II*c*) comparable with those obtained in rabbit serum (Table I).

No activation of protease was observed in rat serum after incubation with New Zealand agar at a concentration of 830 μg./ml. for 3 min. The same sample of agar was tested for its ability to release histamine from rabbit blood cells *in vitro* by the method previously described (Haining, 1955). In one experiment, incubation of rabbit blood with agar (100 μg./ml.) for 30 min. gave a plasma histamine level of 1.2 μg./ml. compared with 0.14 μg./ml. of blood for the control sample, whereas a concentration of 710 μg./ml. in a different sample of blood increased the level from 0.25 μg./ml. to 2.2 μg./ml.

Dextran D caused insignificant increases in protease activity in rabbit serum, rat serum or oxalated rabbit plasma even with concentrations as high as 50 mg./ml. (Table II).

TABLE II

PROTEASE ACTIVATION IN SERUM AND PLASMA

System	Conc. of Polysaccharide (μg./ml.)	L-Tyrosine Equivalent (μg./ml. Serum or Dilute Plasma)	No. of Observations	*P*
a Rabbit serum	Control	17·0		
	Dextran sulphate I 100	30·4	5	>0·05
	,, ,, E/1 100	47·4	5	<0·05
	,, ,, D/3 100	55·8	5	<0·01
b Rabbit serum	Control	16·8		
	Dextran D 2,000	16·4	5	
	,, 10,000	18·3	6	
	,, 50,000	21·7	6	>0·05
c Oxalated rabbit plasma	Control	6·6		
	Dextran sulphate D/3 4	15·4	5	<0·02
	,, ,, 20	24·1	5	<0·01
	,, ,, 100	30·9	5	<0·05
d Oxalated rabbit plasma	Control	6·6		
	Dextran D 2,000	6·3	5	
	,, 10,000	9·5	5	>0·05
	,, 50,000	9·9	4	>0·05
e Pooled rat serum	Control	4·0		
	Dextran sulphate D/3 20	9·9	5	>0·05
	,, ,, 100	19·0	4	<0·01
	,, ,, 500	5·2	5	>0·05
f Pooled rat serum	Control	2·4		
	Dextran D 2,000	0·7	3	
	,, 10,000	5·3	4	>0·05
	,, 50,000	13·3	4	>0·05
g Guinea-pig serum	Control	96		
	Dextran sulphate D/3 100	172	10	<0·05
	,, ,, ,, 700	80	7	>0·05

DISCUSSION

There is considerable indirect evidence to suggest that histamine release may in some circumstances depend upon a proteolytic mechanism (Ungar, 1953; Rocha e Silva, 1950). A close correlation exists between histamine release and protein breakdown both during antigen-antibody reactions *in vitro*, and during incubation of normal tissue with Tween 20, octadecylamine, morphine sulphate, and compound 48/80. Furthermore, these agents and also peptone and a variety of polysaccharides have been shown to activate the proteolytic enzyme present in guinea-pig serum (Ungar, Damgaard, and Hummel, 1953; Ungar and Damgaard, 1955). In the present experiments, the parallelism between histamine release and protease activation by dextran sulphate is sufficiently close to suggest a relation between the two phenomena.

It is difficult to explain the inactivity of dextran D as a protease activator. Since dextran and dextran sulphate are so closely related structurally, it is unlikely that the mechanisms by which they release histamine differ to any great extent. Failure to activate protease by a dextran known to be capable of releasing histamine may therefore indicate that the activation of protease by dextran sulphate is unrelated to histamine release. However, it is significant that agar—which like dextran D also released histamine when incubated with rabbit blood and yet failed to activate rabbit or rat serum protease—does activate protease in guinea-pig serum (Ungar and Mist, 1949). Humphrey and Jaques (1955) found that rabbit plasma incubated with agar developed proteolytic activity towards denatured haemoglobin. The possibility therefore remains that failure to demonstrate activation of protease in the present experiments can be attributed to failure to employ optimum conditions.

The significance of the inhibitory effect of high concentrations of dextran sulphate on histamine release and protease activation is not clear. Similar effects have been observed in other *in vitro* anaphylactic and anaphylactoid reactions (Ungar *et al.*, 1953), in the precipitin reaction between antigen and antibody, and in the precipitation which occurs when increasing concentrations of dextran sulphate of high molecular weight are added to a solution of fibrinogen (Walton, 1952). Furthermore, when excess antigen is incubated with tissue from a sensitized animal, the amount of protease activation is diminished (Ungar *et al.*, 1953; Geiger, 1952).

Summary

1. Certain fractions of dextran sulphate activated protease when incubated with oxalated rabbit plasma or the sera of rabbits, rats and guinea-pigs. No protease activation was observed with dextran (approximate mol. wt. 220,000).

2. The samples of dextran sulphate which activated protease were the same as those which released histamine from rabbit blood cells; they had molecular weights of approximately 40,000 and 440,000. A sample with a molecular weight of 14,000 was inactive.

3. When dextran sulphate (approximate mol. wt. 440,000) was incubated with rabbit serum there was an optimal concentration for maximum activation of protease. The range of concentrations over which protease activation occurred was within that previously found to cause histamine release in rabbit blood.

I wish to thank Professor A. C. Frazer for his continued interest in this work, Dr. P. B. Marshall for helpful discussions, and Miss H. Turberfield for technical assistance. Dextrans and dextran sulphates were generously supplied by Dr. K. W. Walton and Dr. C. R. Ricketts.

This work was carried out during the tenure of a grant from the Colonial Products Research Council.

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Brit. J. Pharmacol. (1956), **11**, 111.

A DELAYED SLOW CONTRACTING EFFECT OF SERUM AND PLASMA DUE TO THE RELEASE OF A SUBSTANCE RESEMBLING KALLIDIN AND BRADYKININ

BY

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(RECEIVED SEPTEMBER 1, 1955)

During experiments on a delayed slow contracting effect of serum and plasma on the isolated guinea-pig ileum, it was found that this activity was not due to a substance originally present, but to one released or formed in the isolated organ bath during the test (Schachter, 1955). In the present experiments this phenomenon has been further analysed.

The incubation of Tyrode-diluted serum for 2–20 min. results in the release of a smooth-muscle stimulant; the onset and rate of contraction are therefore more rapid when the fluid bathing the test preparation is replaced by pre-diluted serum than when dilution of the serum occurs within the bath. The properties of the substance released resemble those of kallidin, which was originally called substance DK (Werle, Götze and Keppler, 1937; Werle and Grunz, 1939; Werle and Berek, 1950) and bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949; Prado, Beraldo and Rocha e Silva, 1950; Andrade, Diniz and Rocha e Silva, 1953). A provisional hypothesis is that dilution releases a smooth-muscle stimulant from serum through activation of serum kallikreinogen (see Frey, Kraut and Werle, 1950), resulting in the release of kallidin.

Most experiments were carried out with dialysed ox and guinea-pig serum, but a similar phenomenon, though quantitatively less striking, was observed with rat, dog, cat and human serum or plasma. Rabbit and hen serum either failed to exhibit this reaction or gave doubtful results.

Experiments were also carried out on the effectiveness of kallikrein (Padutin), saliva, urine, trypsin, renin, and fibrinolysin in releasing smooth-muscle stimulating agents from serum of different species. Their relative abilities to release muscle stimulating substances from different sera were not parallel. The histamine releasing agents, compound 48/80, egg white, and wasp venom, did not release kallidin or bradykinin on incubation with serum.

METHODS

Serum and Plasma.—Blood was collected in glass vessels and centrifuged at 1,400 *g* for 20 min. Serum or plasma was carefully removed from the cell layer without delay, and either tested or frozen at once in small volumes for future use. Samples were thawed at room temperature or with slight warming when required, and were still active after several months in the frozen state; thawed samples were never re-frozen.

Ox blood was obtained at slaughter and defibrinated; all other sera were obtained from clotted blood. Guinea-pigs were stunned by a blow on the head and blood collected by cutting a jugular vein. Rats, cats, dogs and rabbits were anaesthetized with intravenous or intraperitoneal pentobarbitone sodium (30 mg./kg.) and bled from a carotid artery. Human blood was obtained by venepuncture, and hen blood by cutting a vertebral artery.

When plasma was employed, blood was collected in 1% heparin-saline (1.0 ml./100 ml. blood). Serum or plasma was dialysed, when necessary, in cellophane sacs at 4° C. for 24 hr., against 10–20 volumes of saline, changed 3 times.

Isolated Smooth-muscle Test Preparations.—Isolated organ chambers (which varied in size with different preparations) were made with a constriction (2–3 mm. internal diameter) at the base, so that diffusion of added serum was restricted as much as possible to a definite volume. This precaution reduced variations in the delay and the degree of contraction following the addition of native serum to the chamber.

The stated volumes of the baths represent their fluid content in the presence of the particular test preparation, rather than the total volume; this distinction is important in determining the final concentration of serum in the muscle chamber. The fluid volumes of the chambers while containing the different preparations were approximately: 17 ml. for guinea-pig intestine or uterus, and for rat and cat uterus; 20 ml. for cat intestine; and 40 ml. for dog intestine.

Drugs and Other Agents.—Atropine and mepyramine were used as sulphate and maleate respectively; synthetic 5-hydroxytryptamine creatinine sulphate (weight ex-

pressed as base) was supplied by Abbott Laboratories and 48/80 by Burroughs Wellcome.

Renin, prepared from rabbit kidney, was kindly provided by Dr. W. S. Peart, and ox-serum bradykinin by Dr. M. Rocha e Silva. Ox-serum fibrinolysin, prepared by Dr. E. C. Loomis, was obtained through Dr. G. Ungar. Trypsin and chymotrypsin were crystalline preparations from ox pancreas (Armour). Soya bean and ovomucoid trypsin inhibitors were crystalline preparations (Worthington).

Padutin (Bayer) is a kallikrein preparation from hog pancreas preserved in 0.3% tricresol. It frequently produced a quick, rapidly relaxing contraction of the guinea-pig ileum, and the tricresol depressed the response of the ileum to various stimulants. These effects were eliminated by dialysis of Padutin (against 50–100 volumes of saline at 4° C. for 6 hr.) without affecting its ability to release kallidin. The residue, approximately doubled in volume, was made up to 4 kallikrein units/ml. with saline and used at once, or frozen in small volumes for future use.

Human mixed saliva was obtained after rinsing the mouth with saline. Cat chorda saliva was collected from the submaxillary duct during stimulation of the chorda tympani nerve under chloralose anaesthesia (80 mg./kg.).

Results

Release of a Smooth-muscle Stimulant from Serum by Dilution

Ox Serum.—The addition of 0.5–2.0 ml. dialysed ox serum to a 17 ml. bath (containing atropine and mepyramine) regularly caused a delayed, slow contraction of the guinea-pig ileum. The delay varied from 30 sec. to 3 min., and the contraction reached its peak in 2–6 min. The muscle then gradually relaxed in the presence of the diluted serum. The relaxation rate varied and was often more rapid in the presence of the greater quantities of serum, e.g., 2.0 ml. The largest contractions were obtained with the addition of 1.0–2.0 ml.; this volume of serum was optimal, since greater amounts produced smaller effects. The evidence presented below indicates that this delayed, slow contraction is due, not to a substance already present, but to its formation or release from serum when the latter is diluted in the test bath. (Dialysed ox serum also contains a pre-formed substance of the slow contracting type which becomes evident when 5.0 ml. or more of serum is tested in a 17 ml. bath.)

Diluted ox serum in concentrations of 1/35–1/10, incubated for 5–20 min. at 35° C., produced a marked contraction of the guinea-pig ileum when the bath fluid was replaced by the diluted serum. The total amount of serum in the 17 ml. bath was, as before, 0.5–2.0 ml. The contraction began in about 5 sec. and reached its maximum in about 60 sec.

The release of a smooth-muscle stimulant by dilution of ox serum with Tyrode solution was also demonstrated on isolated preparations of cat and dog intestine, and on guinea-pig, rat and cat

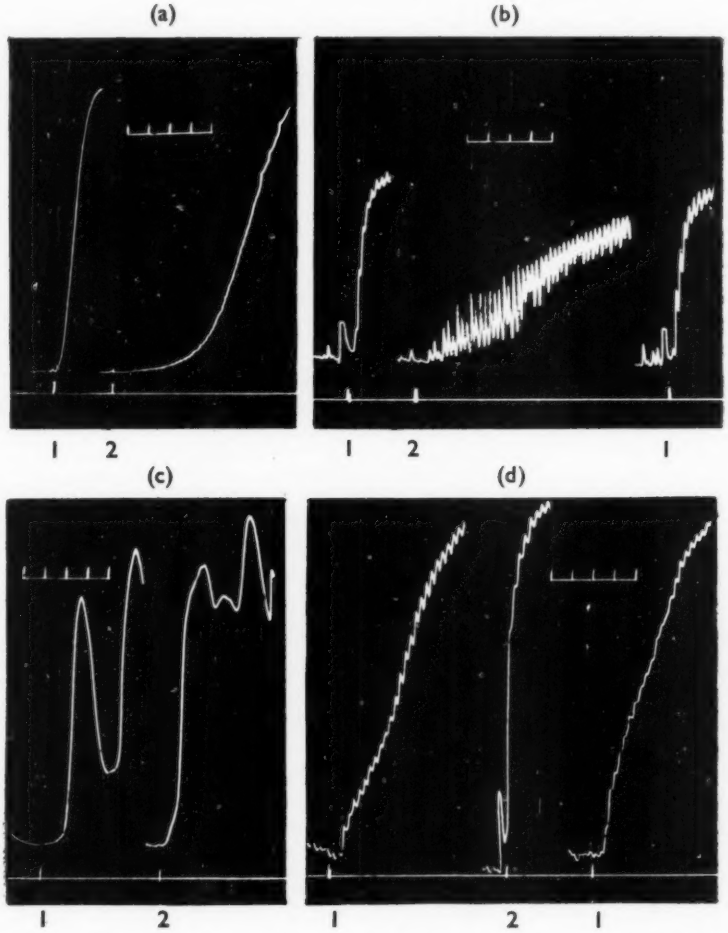

FIG. 1.—Delayed, slow contraction produced by dialysed ox serum; more rapid onset and rate of contraction if serum is diluted and incubated before testing. Bath temp. 35° C., except rat uterus, 28° C. Atropine (0.01 μg./ml.) and mepyramine (0.02 μg./ml.), present throughout. Serum was diluted with Mg-free Tyrode solution and incubated for 6 min. at 35° C. (for rat uterus at 28° C.). Time, 30 sec. (a) Guinea-pig ileum, 17 ml. bath. 1, 17 ml. diluted (0.5/17), incubated serum; 2, 0.5 ml. serum. (b) Dog small intestine, 40 ml. bath. 1, 40 ml. diluted (2/40), incubated serum; 2, 2 ml. serum. (c) Non-oestrous rat uterus, 17 ml. bath. 1, 0.5 ml. serum; 2, 17 ml. diluted (0.5/17), incubated ox serum. (d) Cat small intestine, 20 ml. bath. 1, 1 ml. serum; 2 20 ml. diluted (1/20), incubated serum.

uterus (Fig. 1). Rat uterus and cat intestine were particularly sensitive preparations.

Guinea-pig Serum.—A similar phenomenon, with quantitative differences, was observed with guinea-pig serum or plasma. Thus, guinea-pig serum produced a delayed, slow contraction of the guinea-pig ileum, but the delay of contraction was less than with ox serum, and the effect was usually optimal when only 0.2–0.25 ml. was added to the 17 ml. bath (Fig. 2). Also, as described later, the active substance in guinea-pig serum was released, and also inactivated more rapidly, than that of ox serum; diluted guinea-pig serum was therefore incubated for only 1–5 min. before testing. As with ox serum, this phenomenon was demonstrated with various smooth-muscle preparations.

Other Sera.—Rat, dog, cat, and human serum or plasma also released a smooth-muscle stimulant on dilution. These sera were usually dialysed, since the amounts tested often contained considerable quantities of 5-hydroxytryptamine or other quick contracting agents. The active agent was usually released when serum was incubated for 5–15 min. at the following concentrations: man 1/40–1/20, rat 1/10, dog and cat 1/50–1/15. Since only a limited number of observations was made with these sera, the concentrations found effective are not necessarily optimal. Human and cat serum did not produce effects as pronounced, or as regular after dilution, as did ox and guinea-pig serum; but an effect was usually detectable and was occasionally marked (cf., Fig. 4). With human and cat serum the release could usually be demonstrated by testing diluted, incubated (5–10 min.) serum, even if it was slight or undetectable when native serum was diluted in the test bath itself.

Rabbit and hen serum failed to show definite evidence of release of a smooth-muscle stimulant on dilution. Hen serum contained large amounts (approximately 1.0 μg./ml.) of a substance resembling 5-hydroxytryptamine (see Erspamer and Sala, 1954).

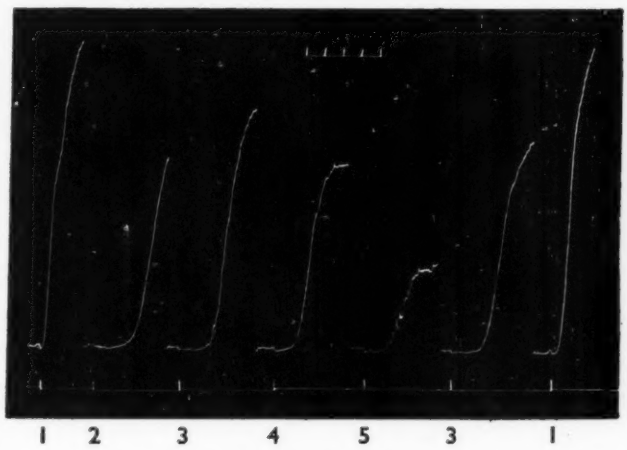

FIG. 2.—Delayed, slow contraction of the isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present) produced by guinea-pig serum; more rapid onset and rate of contraction with serum diluted and incubated before testing. Time, 30 sec. 1, 17 ml. diluted (0.2/17) serum, incubated 2 min.; 2, 0.1 ml. serum; 3, 0.2 ml. serum; 4, 0.5 ml. serum; 5, 0.8 ml. serum.

Properties of the Smooth-muscle Stimulant Released by Dilution of Serum

The properties of the smooth-muscle stimulant released by dilution were mostly studied with ox and guinea-pig serum. The effect was not significantly reduced when the serum was additionally centrifuged at 17,000 *g* for 20 min.; the active substance must therefore be derived from serum and not from blood cells. It is readily distinguishable from acetylcholine, histamine, and 5-hydroxytryptamine both by a slower rate of contraction and subsequent relaxation of the ileum, and by the fact that the contraction is unaffected by atropine, by mepyramine, or by 5-hydroxytryptamine desensitization. It also differs from serum derivatives with smooth-muscle stimulating properties such as the kallikreins, which do not stimulate the guinea-pig intestine or uterus (see Werle, 1955), and from anaphylatoxin, which rapidly desensitizes the test preparation (Rothschild and Rocha e Silva, 1954). It does, however, share many properties with bradykinin and kallidin, both of which are derived from an α_2 serum globulin (van Arman, 1952, 1955; Werle, 1953, 1955).

In addition to producing a slow contraction of the guinea-pig ileum, like kallidin, it contracted the isolated intestine of the cat and dog, and the uterus of the guinea-pig, rat, and cat (Fig. 1). Also, its action on all these test objects paralleled that of a preparation of bradykinin both qualitatively and quantitatively. The slow contractor released by diluted (1/75) guinea-pig serum was usually inactivated in 15–30 min. at 35° C., although sometimes it took longer. A 1/35 dilution of ox serum became inactive in 60–90 min. It is of interest in this connexion that, of all mammalian sera studied, guinea-pig serum is the most effective in inactivating kallidin (Werle and Hambuechen, 1943). The addition of chymotrypsin to diluted serum markedly increased the rate of inactivation of the active substance (Fig. 5). Chymotrypsin and serum also quickly inactivate kallidin and bradykinin (Werle, Kehl, and Koebke, 1950; Rocha e Silva, 1951).

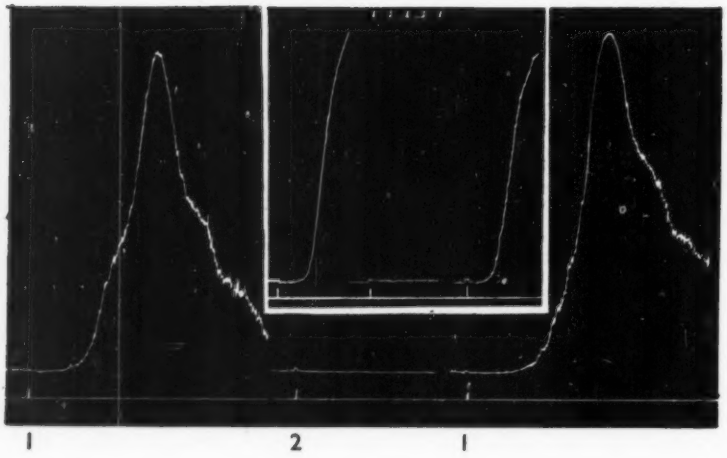

FIG. 3.—Delayed, slow contraction of isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present) produced by ox or guinea-pig serum, and inhibition of this effect by STI. Time, 30 sec. Main figure: 1, 2 ml. dialysed ox serum; 2, 2 ml. dialysed ox serum previously incubated (35° C.) for 1 min. with 0.4 mg. (0.4 ml.) STI. Inset figure: at first and last signals, 0.2 ml guinea-pig serum; at intervening signal, 0.2 ml. guinea-pig serum, but 0.4 mg. (0.4 ml.) STI present in bath.

Inhibition of Release of the Smooth-muscle Stimulant by Soya Bean Trypsin Inhibitor (STI)

Incubation of 0.5–2.0 ml. ox serum, or 0.20–0.25 ml. guinea-pig serum with 0.2–0.5 mg. STI (1 mg./ml.) for 1 min., regularly prevented the release of the smooth-muscle stimulant on subsequent dilution of the serum (Fig. 3). The inhibition of release was equally effective if the sera were diluted with 17 ml. Tyrode solution containing 0.2–0.5 mg. STI, i.e., 12–30 μg./ml. Double this concentration of ovomucoid inhibitor (OI) was ineffective. The addition of STI to diluted ox or guinea-pig serum which had already been incubated did not abolish the action of the released smooth-muscle stimulant.

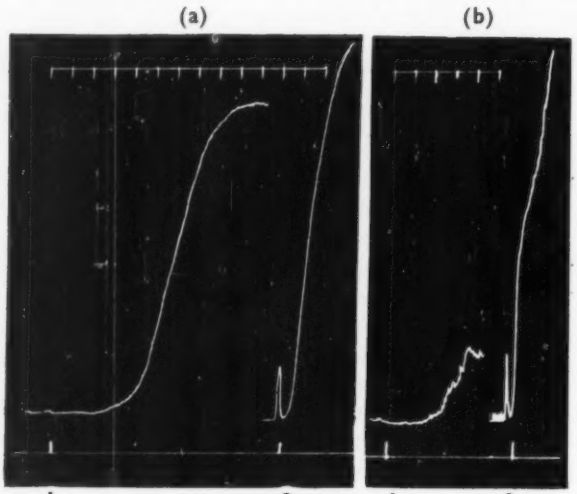

FIG. 4.—Delayed, slow contraction of isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present) produced by dialysed human and cat plasma; more rapid onset and rate of contraction with plasmas diluted with Tyrode solution and incubated (5 min., 35° C.) before testing. Time, 30 sec. (a) 1, 0.4 ml. human plasma; 2, 17 ml. diluted (0.4/17), incubated human plasma. (b) 1, 0.25 ml. cat plasma; 2, 17 ml. diluted (0.25/17), incubated cat plasma.

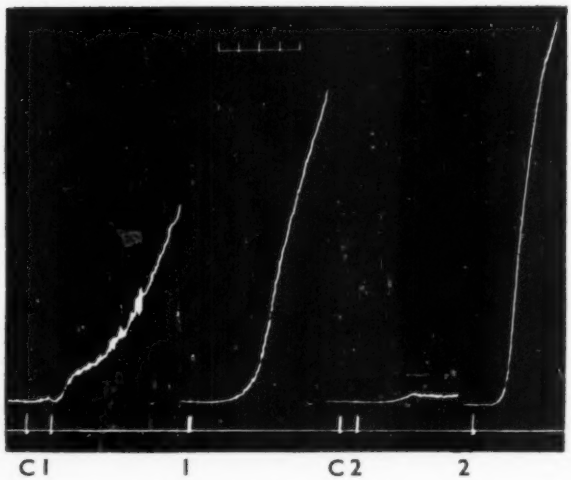

FIG. 5.—Reduced effect of ox and guinea-pig serum on the isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present) in the presence of chymotrypsin. Continuous tracing indicates bath not washed out. Time, 30 sec. C, 0.4 mg. (0.4 ml.) chymotrypsin. 1, 1 ml. dialysed ox serum. 2, 0.2 ml. guinea-pig serum.

However, the addition of STI to diluted guinea-pig serum, previously incubated for a few minutes, did substantially reduce the activity; with diluted ox serum the contraction was either unaffected or only slightly reduced. If, as suggested, dilution activates an agent (sensitive to STI) which releases a smooth-muscle stimulating polypeptide from a serum substrate, then the amount of active polypeptide present at any time would be the resultant of its rate of release and simultaneous inactivation by serum peptidase. Thus, if STI arrested the progressive release, but not the inactivation of the active substance, then a reduced effect, as observed, might be expected, particularly with guinea-pig serum where inactivation is more rapid. The above

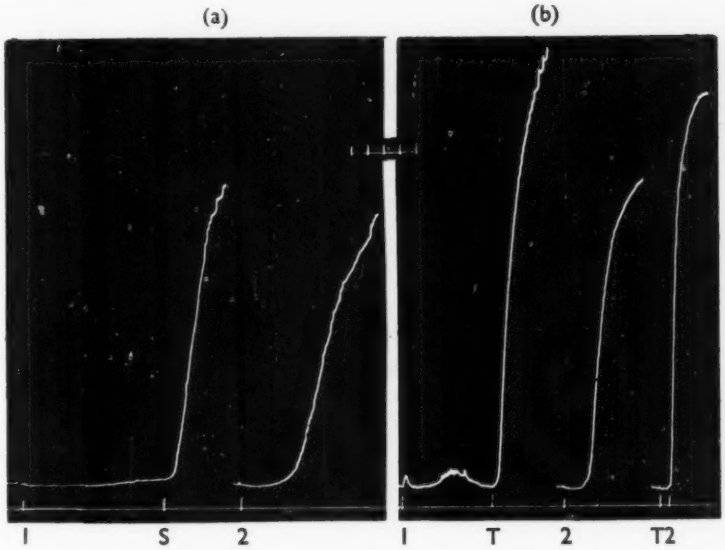

FIG. 6.--Ox or guinea-pig serum heated to 56° C. for 3 hr. fails to produce a delayed slow contraction of the isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present); also, trypsin releases as much bradykinin from heated as from unheated guinea-pig serum. Continuous tracing indicates bath not washed out. Time 30 sec. Intestine desensitized to trypsin between (a) and (b). (a) 1, 0.5 ml. heated, dialysed ox serum; S, 0.2 ml. human saliva; 2, 0.5 ml. dialysed ox serum. (b) 1, 0.25 ml. heated guinea-pig serum; T, 0.3 mg. (0.3 ml.) trypsin; 2, 0.25 ml. guinea-pig serum.

facts, therefore, are not necessarily in disagreement with the hypothesis presented.

The concentration of STI which regularly prevented release of the muscle stimulant by dilution of ox or guinea-pig serum was only occasionally completely effective with human serum, although the effect was always reduced. STI only slightly reduced the release from cat serum. Thus, the relative effectiveness of STI in inhibiting the release of the active substance varies with different sera.

Failure to Release a Smooth-muscle Stimulant by Dilution of Ox and Guinea-pig Serum after Destruction of Kallikreinogen

Since kallikreinogen (kallikrein plus its inactivator) is destroyed by heating serum for 3 hr. at 56° C. (Werle *et al.*, 1937), it was of interest to see whether this treatment affected the release of the kallidin-like substance by dilution. It was indeed observed that ox and guinea-pig serum, heated in this way, released little or no smooth-muscle stimulant when subsequently diluted; such heated serum, none the less, released as much kallidin or bradykinin as unheated serum upon addition of saliva or trypsin (Fig. 6), indicating that heating in this way leaves kallidinogen and bradykininogen unaffected. That kallidinogen remains unaffected by this degree of heating was long ago noted by Werle *et al.* (1937).

It was also of interest to test whether serum exhausted of kallidinogen and bradykininogen would still release a kallidin-like substance on dilution. Several types of experiments were performed to test this possibility. Thus, the incubation of 0.25 ml. guinea-pig serum with 0.25–0.3 mg. trypsin (0.25–0.3 ml.) released large amounts of bradykinin which was inactivated, presumably by peptidase in serum, after about 60 min. at 35° C. This serum now released little or no kallidin on addition of Padutin (1 kallikrein unit in 0.25 ml.), or on dilution. The addition of 0.3 mg. renin (0.3 ml.), however, still released moderate amounts of a slow-contracting substance from the mixture, although the amount released was less than from untreated serum. Conversely, optimally diluted guinea-pig serum, after incubation for 10–60 min., released very little kallidin or bradykinin (by Padutin or trypsin), but the subsequent addition of renin was still moderately effective (Fig. 7).

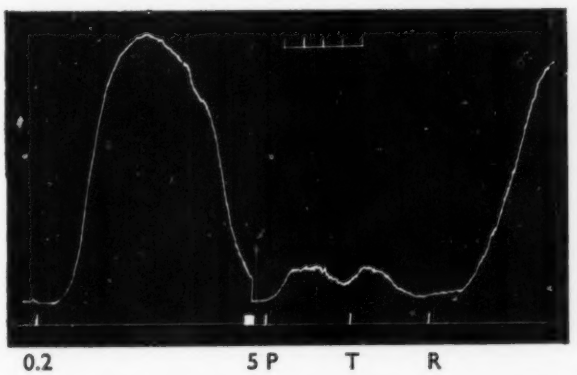

FIG. 7.—After the smooth-muscle stimulant is released by dilution, and inactivated in guinea-pig serum, the release of kallidin and bradykinin by Padutin and trypsin respectively is much reduced; but renin still releases a slow contractor. The longer delay required for renin to release a slow contractor is characteristic. Isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present). Continuous tracing indicates bath not washed out. Time, 30 sec. At 0.2, 0.2 ml. dialysed guinea-pig serum; at 5, kymograph stopped for 5 min. P, Padutin (0.25 ml., 1 kallikrein unit); T, 0.3 mg. trypsin (0.3 ml.); R, 0.3 mg. renin (0.3 ml.).

Incubation of 0.5 ml. diluted ox serum for long periods, unlike guinea-pig serum, still released large amounts of kallidin on subsequent addition of saliva. Similarly, saliva still released large amounts of kallidin from diluted human serum. This is perhaps because the kallidinogen and bradykininogen of guinea-pig serum are largely exhausted by optimal dilution, whereas those of ox and human serum are only reduced.

Relative Effectiveness of Padutin, Trypsin, Fibrinolysin, Renin, Saliva and Urine, in Releasing Smooth-muscle Stimulants from Different Mammalian Sera

During the above experiments it was observed that various agents releasing kallidin or bradykinin possessed this ability to a strikingly different degree with different sera as substrates (Fig. 8). For example, Padutin was a potent releasing agent from guinea-pig serum, but completely ineffective, under the same conditions, with human, cat, or rabbit serum; on the other hand, human mixed saliva, and particularly cat chorda saliva, were potent releasing agents with human, cat, and rabbit serum, but ineffective with guinea-pig serum. These results indicate a degree of species specificity of kallikrein and renin for their substrates—kallidinogen and hypertensinogen (Table I). Relatively high concentrations of a purified bovine fibrinolysin preparation (5 mg./ml.) were required to release significants amounts of a smooth-muscle stimulant from ox, human, and guinea-pig serum.

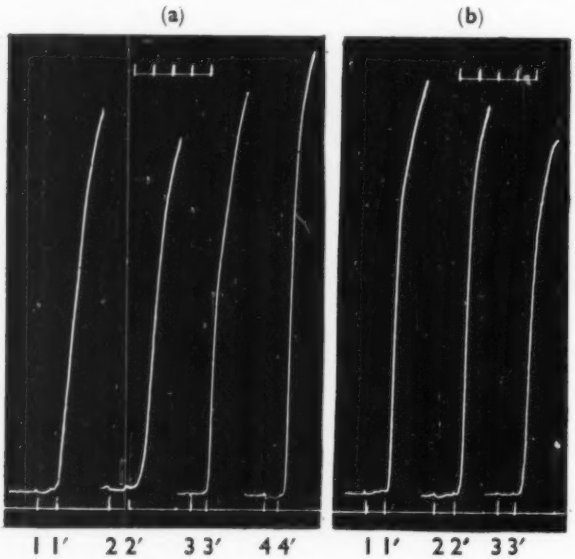

FIG. 8.—Difference in relative ability of Padutin, saliva and urine in releasing a kallidin- or bradykinin-like substance from serum of different species; also, failure of wasp venom, egg white, and 48/80 to release a smooth-muscle stimulant from human serum. Isolated guinea-pig ileum (17 ml. bath; atropine and mepyramine present). Dialysed serum or plasma (0.7 ml.) incubated with various agents at 35° C. for 2 min. Continuous tracing indicates bath not washed out. Time, 30 sec. (a) 1, guinea-pig serum+0.5 ml. human urine; 1′, Padutin (1 kallikrein unit); 2, ox serum+0.5 ml. human urine; 2′, 0.2 ml. human saliva; 3, guinea-pig serum+0.5 ml. guinea-pig urine; 3′, Padutin (1 unit); 4, cat plasma+Padutin (1 unit); 4′, 0.1 ml. cat chorda saliva. (b) 1, human serum+0.2 mg. (0.3 ml.) dialysed wasp venom; 1′, 0.2 ml. human saliva; 2, human serum+10% hen egg white (0.3 ml.); 2′, 0.1 ml. cat chorda saliva; 3, human plasma+0.03 mg. 48/80 (0.3 ml.); 3′, 0.5 ml. human urine.

STI and OI were also tested for their ability to prevent release of the smooth-muscle stimulants from serum by these various agents. The releasing abilities of human saliva and of urine were unaffected by incubation with STI or OI (1.0 mg./ml.) for 1–2 min. The active releasing agents in these instances are therefore, in all probability, the kallikreins of saliva and urine, respectively (for literature on kallikrein in saliva, see Werle and von Roden, 1936; Ungar and Parrot, 1936; Guimarais and Tavares, 1942; Werle and Maier, 1952). The releasing ability of fibrinolysin was completely inhibited by STI (0.5 mg./ml.), but unaffected by the same amount of OI. The sensitivity of fibrinolysin to inhibitors is therefore similar to that of the serum kallikrein prepared by the acetone method (Werle and Maier, 1952).

TABLE I

RELATIVE ABILITY OF VARIOUS AGENTS TO RELEASE A SMOOTH-MUSCLE STIMULANT FROM DIFFERENT MAMMALIAN SERA AFTER 2 MIN. INCUBATION AT 35° C.

Releasing Agent and Its Source	Serum (0·7 ml. Dialysed)				
	Ox	Guinea-pig	Man	Cat	Rabbit
Padutin—hog pancreas (1.0 kallikrein unit; 0.25 ml.)	+	++++	−	−	−
Trypsin—ox pancreas (0.3 mg., 0.3 ml.)	+	++++	++	n.t.	n.t.
Renin—rabbit kidney (0.3 mg., 0.3 ml.)	++++	++++	−	+++	++++
Saliva—man (0.2 ml.)	++++	−	++++	++	++
Saliva—cat, chorda (0.1 ml.)	++++	−	++++	++++	++++
Urine—man (0.5 ml.)	−	−	++++	−	n.t.
Urine—guinea-pig (0.5 ml.)	−	−	−	−	−

+ to ++++, relative amount of smooth-muscle stimulant released; −, no release detected; n.t., not tested.

The observation that the administration of histamine liberators to animals results in the appearance of a slow-contracting substance in plasma (Paton, 1951; Jaques and Schachter, 1954) suggested the possibility that these agents might release such a substance from serum. Compound 48/80 (Paton, 1951), egg white (Schachter and

Talesnik, 1952) and wasp venom (Jaques and Schachter, 1954), however, all failed to release kallidin or bradykinin on incubation with human serum (Fig. 8).

Discussion

The present results are of interest from a practical standpoint, since they indicate the possibility of a potent smooth-muscle stimulant being formed under conditions which exist in many biological assays. The possibility that errors of interpretation may occur for this reason during assay of different body fluids, as well as with plasma or serum, must be considered.

The process whereby dilution releases a smooth-muscle stimulant from serum is of considerable interest from a theoretical point of view. It is not surprising that new properties of serum result from dilution, since dilution is known to dissociate large molecular complexes such as trypsin-antitrypsin (Hussey and Northrop, 1923) and toxin-antitoxin (Glenny and Barr, 1932). Dilution of serum also activates profibrinolysin (MacFarlane and Pilling, 1946); results in the appearance of substances in guinea-pig serum which increase capillary permeability, and "bind" small amounts of histamine (MacKay, Miles, Schachter and Wilhelm, 1953; Miles and Wilhelm, 1955); and produces an anaphylatoxin in rat serum (Rothschild and Rocha e Silva, 1954). The observation that human serum releases a substance resembling bradykinin on first contact with glass (Armstrong, Keele, Jepson and Stewart, 1954) is of particular interest. Again, contact with glass has been reported to activate (independently of platelet disruption) many of the large molecular serum clotting factors, e.g., a thromboplastin precursor, prothrombinogen, and proconvertin (see Rapaport, Aas and Owren, 1955). Further work is desirable to determine whether the similar smooth-muscle stimulants released from serum on first contact with glass, and by dilution, are end-results of the same or different mechanisms.

How does dilution of serum or plasma result in the release of a substance resembling kallidin and bradykinin? The present observations do not justify more than a speculative interpretation based on the observations described and on the properties of known substances and their precursors in serum. Of the known agents derived from serum, and capable of releasing a smooth-muscle stimulant similar to the one released by dilution, the serum kallikreins stand out as likely possibilities, particularly kallikrein, prepared by the acetone method, whose kallidin releasing action is also sensitive to STI but not to OI (Werle and Maier, 1952). Furthermore, the release of a kallidin-like substance by dilution failed to occur when ox or guinea-pig serum was first heated for 3 hr. at 56° C.; kallikreinogen, the inactive precursor of kallikrein, is likewise destroyed by this treatment (Werle *et al.*, 1937). Also, guinea-pig serum exhausted of kallidinogen by Padutin (or of bradykininogen by trypsin) failed to release a smooth-muscle stimulant on subsequent dilution. The properties of the latter agent correspond to those of kallidin and bradykinin in its instability in serum, susceptibility to chymotrypsin, and action on various test preparations. All the above observations are therefore consistent with the hypothesis that dilution of serum releases kallidin through the activation of kallikreinogen. Purified kallikrein preparations release detectable amounts of kallidin when only 0.003 μg. is incubated with serum (Werle *et al.*, 1937). This order of activity is about a thousandfold greater than observed in the present experiments for the bradykinin-releasing activity of crystalline trypsin, and far greater still than for a purified preparation of fibrinolysin. Also, in the present experiments, as little as 0.01 ml. cat chorda saliva, most probably through the action of salivary kallikrein, released detectable amounts of a kallidin-like substance from cat serum.

Thus, the dilution of serum might simply dissociate the kallikrein-inactivator complex, or it might activate serum proteases (like fibrinolysin), which in turn might release kallikrein. It has, in fact, been shown recently that trypsin releases kallikrein from its inactive complex in serum (Werle, Forell, and Maier, 1955). The possibility also arises, therefore, that the release of bradykinin from serum by trypsin is entirely due to such an activation. This is not so, however, since in the present experiments trypsin released as much bradykinin from heated serum (which destroyed kallikreinogen) as it did from unheated serum.

The fact that exhaustion of kallidinogen of guinea-pig serum by Padutin (or of bradykininogen by trypsin) abolished the subsequent release of a kallidin-like substance by dilution of the same serum suggests that the smooth-muscle stimulants are released from a common substrate in these instances; however, since renin released moderate amounts of a slow contracting substance from serum previously exposed to all the above procedures, it would appear that hypertensinogen is either distinct from kallidinogen and bradykininogen, or the same protein, but split at a different site. These conclusions regarding the specificity of substrates are, however, made with reservation, since the possibility cannot be excluded that the activities of fibrinolysin

and renin are to some degree due to contamination with kallikrein, or those of Padutin to contamination with trypsin. A definite answer to this question can only be obtained by further experiments with highly purified releasing agents and their serum substrates.

Summary

1. Dilution of ox, guinea-pig, rat, dog, cat and human serum or plasma releases a smooth-muscle stimulating agent resembling kallidin and bradykinin. The optimal dilution, the amount of muscle stimulant released, and its rate of inactivation vary with the sera of different species. The release of this substance is demonstrable when dilution occurs in the test bath, but the effect is more striking if diluted serum is incubated for a definite period and then tested.

2. The release of the kallidin-like substance by dilution of ox or guinea-pig serum is greatly reduced or abolished: (a) in the presence of soya bean trypsin inhibitor, (b) if serum is heated before dilution at 56° C. for 3 hr. to destroy kallikreinogen.

3. The smooth-muscle stimulant released by dilution resembles kallidin and bradykinin in that: (a) it contracts, in a characteristic way, the guinea-pig, cat, and dog intestine, and the guinea-pig, rat, and cat uterus ; (b) it is inactivated by serum and by chymotrypsin.

4. A comparison of the relative ability of various agents which release smooth-muscle stimulants from serum showed that this property was not parallel with sera of different species. The results indicate a degree of species specificity of kallikrein and renin for their serum substrates.

5. Rabbit and hen serum failed to release a smooth-muscle stimulant on dilution. Compound 48/80, egg white, and wasp venom did not release kallidin or bradykinin from serum.

6. The hypothesis is suggested that dilution of serum releases kallidin through activation of kallikreinogen.

I am grateful to Professor G. L. Brown, F.R.S., for his help in putting facilities at my disposal for carrying out this work. I wish, also, to thank Miss Doris Major for able technical assistance.

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STUDIES ON MAMMALIAN HISTIDINE DECARBOXYLASE

BY

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(RECEIVED SEPTEMBER 12, 1955)

Histamine is present in most mammalian tissues, but its mode of formation is still not clear. According to Blaschko (1945) there are two main theories: (1) Histamine is a vitamin, formed outside the body by bacterial decarboxylation of dietary histidine in the alimentary tract. (2) Histamine is a metabolite, formed from circulating histidine by the histidine decarboxylase present in some tissues of the body.

That bacteria form histamine by decarboxylation of histidine is well known (Ackermann, 1910, 1911; Berthelot and Bertrand, 1912; Mellanby and Twort, 1912; Kendall and Gebauer, 1930; Matsuda, 1933; Gale, 1940; Epps, 1945). Gale (1953) showed that the bacterial enzyme had several important differences from the other amino acid decarboxylases which had been studied.

The occurrence of a mammalian histidine decarboxylase was demonstrated by Werle (1936) and by Holtz and Heise (1937a). They showed that, after rabbit kidney had been incubated with histidine in buffered Tyrode solution, histamine was present in the supernatant fluid obtained. Werle and co-workers (Werle, 1936, 1940, 1942; Werle and Herrmann, 1937; Werle and Krautzun, 1938; Werle and Heitzer, 1938; Werle and Daumer, 1940; Werle and Koch, 1949), together with Holtz and co-workers (Holtz and Heise, 1937a, 1937b; Holtz, Heise and Spreyer, 1938; Holtz, Credner and Reinhold, 1939; Holtz and Credner, 1941, 1944) carried out extensive investigations upon the distribution of this enzyme in various tissues of different animal species and investigated many of its physical and chemical properties. They were unable to isolate the enzyme, though Werle and Heitzer (1938) claim to have achieved a partial purification. More recently, evidence of a mammalian histidine decarboxylase has been obtained by Schayer (1952), who injected guinea-pigs with ^{14}C-labelled histidine and histamine. But these studies do not conclusively demonstrate the general occurrence of an enzyme capable of decarboxylating histidine in all mammals, as the experiments were confined to a limited range of mammalian species.

The properties and the distribution in laboratory animals of mammalian histidine decarboxylase, together with the distribution of histaminase and histamine, have been reinvestigated in the hope of clarifying our knowledge of the role of histamine in the organism.

METHODS

Formation of Histamine from Histidine by Mammalian Tissues

Rabbit kidneys, which are a rich source of histidine decarboxylase, were placed in 0.9% w/v NaCl, freed from all extraneous tissue, cut small and minced in a Latapie mincing machine. Where a tissue extract was required, the minced kidney was ground for 10 min. in a porcelain mortar with 1 g. silver sand and 10 ml. 0.9% w/v NaCl/g. tissue. This was centrifuged at 2,500 *g* for 10 min. and the supernatant fluid extract removed for incubation.

Generally 0.4 g. minced tissue in 6 ml. Tyrode solution was used as a source of the enzyme. Occasionally this was replaced by 4 ml. tissue extract plus 2 ml. Tyrode solution. The mixture of tissue and Tyrode solution was placed in a 25 ml. conical flask, 3 ml. M/5-Na_2HPO_4 (*p*H 8.2) and 1 ml. neutralized histidine (15 mg./ml.) were added, and the mixture incubated at 38° C. for 3 hr. with mechanical agitation.

After incubation, 1N-HCl was added from a microburette until a *p*H of 5.5–5.9 was obtained. The mixture was boiled for 1 min. to coagulate proteins and prevent further enzyme action. After cooling, the contents of the flask were filtered through Whatman No. 1 paper.

The histamine content of the filtrate was assayed on the guinea-pig's isolated ileum suspended in a 2 ml. bath of aerated Tyrode solution, with atropine, at 34° C.

Duplicate experiments were always carried out on each tissue mince or extract, and often the experiments were repeated with tissue from another animal. Blanks were carried out to estimate the histamine present in the incubation mixtures in the absence of the tissue, and again in the absence of the histidine solution.

In reporting the results, the average histamine content is given. The largest discrepancy between duplicates among 200 estimates was less than 20%.

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Potentiation or Inhibition of Histidine Decarboxylase

In these experiments 1 ml. Tyrode solution in the standard incubation mixture was replaced by 1 ml. aqueous solution of the test substance before incubation, and 1 ml. distilled water was added after incubation (T). To estimate histamine formation in the absence of the test substance, and to demonstrate any effect which the latter might have on the gut, a control incubation (C_1), in which 1 ml. distilled water was added before incubation and 1 ml. test solution was added after incubation, was carried out. Any difference observed between the results of the test incubation and the control (C_1) must therefore be due either to an effect of the test substance on the enzyme, or to the test substance causing a direct release of histamine from the tissue.

To determine if there was histamine release, a second control mixture (C_2) was prepared, in which 1 ml. distilled water was added in place of 1 ml. histidine solution.

The percentage inhibition or potentiation of the histidine decarboxylase was calculated from the formulae:

$$\% \text{ Inhibition} = 100 - \left(\frac{T - C_2}{C_1}\right) \times 100$$

$$\% \text{ Potentiation} = \frac{T - C_2}{C_1} \times 100$$

Inhibition of the Histaminase Activity of Cat Kidney

Cat kidney was used as a source of histaminase. An extract containing 20 mg. fresh tissue/ml. was prepared by the method described for rabbit kidney. Incubation mixtures (T') consisted of 4 ml. kidney extract+1 ml. Tyrode solution+3 ml. M/3-sodium phosphate buffer pH 7.2 (glass electrode)+1 ml. histamine solution containing 20 μg. base/ml.+1 ml. solution of test substance. The mixtures were incubated for 1 hr. at 38° C., after which 1 ml. distilled water was added, and the histamine content determined, after acidification and boiling. This gave a measure of the histamine destruction in the presence of the test substance. A control sample (C'_1) in which the test substance was omitted during the incubation period, but was added later, gave an estimate of the destruction of histamine in the absence of the test substance. A second control sample (C'_2), containing boiled kidney extract, gave the amount of histamine in the absence of enzyme activity. The percentage decrease of histaminase activity was calculated from the formula:

$$\% \text{ Decrease of activity} = \frac{T' - C'_1}{C'_2 - C'_1} \times 100$$

Inhibition of Histaminase Activity of a Hog Kidney Preparation

The experiment on the histaminase activity of cat kidney was repeated with a different histaminase preparation. This was made by shaking 7.5 mg. purified acetone dried powder of hog kidney with 20 ml. M/3-sodium phosphate buffer of pH 7.2.

Tissue Distribution of Histidine Decarboxylase, Histaminase, and Histamine

The histidine decarboxylase content of the tissue under test was determined by mincing the tissue and incubating it with histidine in buffered Tyrode solution for 3 hr. in the presence of 0.1 ml. 10^{-2} aminoguanidine solution and 1 drop (20 mg.) benzene. Otherwise the procedure was similar to that described for rabbit kidney tissue.

The histaminase content of the tissue was determined by incubating the minced tissue with 1 ml. histamine solution (20 μg./ml.) in buffered Tyrode solution. The procedure was similar to that described for cat kidney tissue, except that the mixture was incubated for 3 hr. Histaminase activity was estimated from the sum: histamine added plus histamine already present in the tissue, minus histamine found after incubation.

The histamine content of the minced tissue was obtained from samples which were added to Tyrode solution, acidified and boiled in the usual manner.

Cellular Distribution of Histidine Decarboxylase, Histaminase, and Histamine

Minced rabbit kidney was used as the source of histidine decarboxylase, minced rabbit lung as the source of histaminase and histamine. These were homogenized in a Potter–Elvjerheim homogenizer with 0.25M-sucrose solution. The homogenized mixtures were then subjected to differential centrifugation, as described by Schneider and Hogeboom (1950). Each homogenate was centrifuged at 2,500 g for 10 min.; this deposits unbroken cells, nuclei and large cell fragments. The kidney extracts were then centrifuged at 37,000 g for 20 min., to deposit the mitochondria, and finally at 90,000 g for 60 min. to deposit the microsomes. The lung extracts were centrifuged at 37,000 g for 60 min. in order to deposit all cell fragments. The final supernatant fluid

TABLE I

HISTAMINE PRODUCTION FROM HISTIDINE BY RABBIT KIDNEYS IN THE PRESENCE OF HISTIDINE AFTER INCUBATION FOR 3 HR. AT 38° C.

Each result is the mean of duplicate incubations.

Rabbit Number	Histamine Formed (μg./g.) with:		Histamine Formed μg./g. Wet Tissue
	Histidine Present	Histidine Absent	
1	17·1	0·1	17·0
2	5·3	0·1	5·2
3	17·6	0·8	16·8
4	22·8	0·8	22·0
5	3·6	0·2	3·4
6	1·4	0·2	1·2
7	11·8	0·5	11·3
8	11·8	<0·1	11·8
9	22·8	0·5	22·3
10	64·0	1·0	63·0
11	31·5	0·5	31·0
12	33·2	0·2	33·0
13	12·5	0·5	12·0
14	20·8	<0·1	20·8
15	2·3	<0·1	2·3
16	7·2	0·2	7·0
17	5·5	<0·1	5·5
18	6·0	0·2	5·8
19	3·5	0·1	3·4
20	5·2	0·2	5·0

TABLE II

INHIBITION OF THE HISTIDINE DECARBOXYLASE ACTIVITY OF RABBIT KIDNEY BY VARIOUS SUBSTANCES

Each result is the mean of duplicate incubations

Concentration:	10^{-2}M			10^{-3}M			10^{-4}M		
Substance	T–C_2	C_1	% Inhibition	T–C_2	C_1	% Inhibition	T–C_2	C_1	% Inhibition
B_1 pyrimidine	6·1	6·5	nil	7·9	8·2	nil	7·8	8·1	nil
Putrescine hydrochloride	7·5	7·0	nil	3·4	3·3	nil	3·3	3·2	nil
Cadaverine dihydrochloride	10·4§	10·7§	nil	9·6	9·4	nil	9·2	9·3	nil
Agmatine sulphate	21·4§	20·8	nil	20·0§	20·0§	nil	18·0§	18·2§	nil
Glyoxaline				5·9	6·1	nil	6·1	6·0	nil
Guanidine carbonate				6·7	6·4	nil	6·7	6·4	nil
Methylguanidine sulphate				5·9	5·9	nil	6·1	5·9	nil
Asym. dimethylguanidine sulphate				6·7	6·4	nil	6·2	6·2	nil
Aminoguanidine bicarbonate				3·7	6·1	40	6·4	6·4	nil
*Iproniazid ("Marsilid")				1·2	8·8	86	7·0	8·5	17
† Girard's reagent "T"				2·1	5·9	66	4·8	6·0	21
‡ Girard's reagent "P"				1·7	6·0	72	3·9	5·9	32
Hydrazine sulphate				nil	6·1	100	nil	6·0	100

*=1-*iso*Nicotinoyl-2-*iso*propylhydrazine. †=Trimethylammonium acetohydrazide chloride. ‡=Pyridinium acetohydrazide chloride.
§=Potentiates responses of histamine on gut.

contained only substances in solution or colloidal particles. Each supernatant fluid extract and residue was tested for histidine decarboxylase and histaminase activity and histamine content.

RESULTS

Normal Formation of Histamine from Histidine by Rabbit Kidneys

The amounts of histamine formed by the kidneys of 20 rabbits are given in Table I; further results will be found in Table V.

The results show the wide variation of histidine decarboxylase activity in the kidneys of different rabbits, the average being 15.0 μg./g. wet tissue within the limits 1.4–63.0. From these results, it will be obvious that no valid comparisons can be made unless in each experiment the test and blank incubations contain tissue from the same animal.

Substances Potentiating or Inhibiting Histidine Decarboxylase

Several substances were incubated with rabbit kidney tissue and histidine, and the results compared with those obtained in the absence of the test substance. The difference between the results gives a measure of the potentiating or inhibitory activity of the substance. In order to demonstrate the presence of histidine decarboxylase in the presence

TABLE III

HISTAMINE (μG.) IN INCUBATION MIXTURES, AFTER INCUBATION WITH CAT KIDNEY EXTRACTS AND A TEST SUBSTANCE

The % inhibition of histamine destruction is also given. Each result is the mean of duplicate incubations.

Concentration:	C'_2 μg. Hist-amine	C'_1 μg. Hist-amine	10^{-7}M		10^{-6}M		10^{-5}M		10^{-4}M		10^{-3}M	
Test Substance			T'	% Inhibition	T'	% Inhibition	T'	% Inhibition	T'	% Inhibition	T'	% Inhibition
Iproniazid	20·7	2·5	2·4	*0*	2·4	*0*	2·4	*0*	3·9	*8*	11·4	*49*
Glyoxaline	20·0	1·0	0·7	*0*	—	—	—	—	—	—	—	—
	20·7	2·5	—	—	7·8	*29*	—	—	—	—	—	—
	19·3	9·4	—	—	—	—	14·7	*53*	19·3	*100*	18·0	*87*
Methylguanidine sulphate	20·0	1·0	1·1	*0*	—	—	—	—	—	—	—	—
	20·7	2·5	—	—	5·8	*18*	—	—	—	—	—	—
	19·3	9·4	—	—	—	—	13·6	*42*	16·8	*75*	19·3	*100*
Asym. dimethylguanidine sulphate	20·0	1·0	1·0	*0*	—	—	—	—	—	—	—	—
	20·7	2·5	—	—	7·3	*26*	—	—	—	—	—	—
	19·3	9·4	—	—	—	—	14·7	*53*	17·9	*86*	19·3	*100*
Aminoguanidine bicarbonate	20·0	1·0	8·1	*32*	10·5	*50*	20·0	*100*	20·0	*100*	20·0	*100*
Guanidine carbonate	20·0	1·0	1·1	*0*	1·3	*2*	—	—	—	—	—	—
	19·3	9·4	—	—	—	—	10·3	*9*	12·6	*32*	16·8	*76*
Hydrazine sulphate	20·7	2·5	15·1	*69*	19·6	*94*	20·4	*99*	20·8	*100*	20·7	*100*
Girard's reagent "P"	20·7	2·5	2·4	*0*	4·9	*13*	11·5	*50*	15·8	*73*	20·6	*100*
Girard's reagent "T"	15·0*	1·4	1·4	*0*	2·6	*9*	4·7	*22*	10·3	*59*	13·9	*83*
B_1 pyrimidine	20·0	1·6	4·1	*13*	5·4	*20*	10·8	*50*	16·0	*76*	18·0	*96*
Putrescine hydrochloride	20·0	1·6	1·6	*0*	1·8	*0*	2·6	*5*	4·5	*15*	11·0	*51*
Cadaverine dihydrochloride	20·0	1·6	1·6	*0*	1·6	*0*	1·6	*0*	5·3	*20*	7·4	*31*
Agmatine sulphate	20·0	1·6	1·5	*0*	1·8	*0*	5·2	*15*	9·1	*40*	16·0	*76*

* 15 μg. histamine added.

of histaminase a search was made for an antihistaminase which would fail to inhibit, and if possible potentiate, histidine decarboxylase. Several known inhibitors of histaminase were investigated, such as putrescine (butane-1 : 4 diamine), and cadaverine (pentane 1 : 5 diamine), previously tested for antihistaminase activity by Werle (1942); B_1 pyrimidine (6 - amino - 5 - aminomethyl - 2 - methylpyrimidine), agmatine (1-amino-4-guanidobutane), guanidine, methyl guanidine, and dimethyl guanidine, all of which were tested by Arunlakshana, Mongar, and Schild (1954). Schuler (1952) tested many compounds as histaminase inhibitors amongst which the most active were amino-guanidine and hydrazine sulphate. Both these were included as well as Girard's reagent "P" (pyridinium-aceto-hydrazide chloride) and Girard's reagent "T" (trimethylamino-aceto-hydrazide chloride), since, from the early work of Werle and his co-workers, they appeared to be likely to behave as selective histaminase inhibitors.

Table II shows that, with the exception of iproniazid (1-*iso*nicotinoyl-2-*iso*propylhydrazine), Girard's reagents "P" and "T," and hydrazine sulphate (which caused marked enzyme inhibition), none of the substances tested inhibited histidine decarboxylase in the concentrations used. It also shows that none of these substances potentiated histidine decarboxylase. Those compounds which had no inhibitory action on histidine decarboxylase were then tested for inhibitory activity on histaminase, with the object of finding a substance which, at a given concentration, would inhibit histaminase completely without affecting histidine decarboxylase.

In Table III the effects of various chemicals upon histaminase activity are given. These results are summarized in a combined table (Table IV) which also includes the effects of these substances upon histidine decarboxylase (see Table II). Methyl guanidine sulphate (10^{-3}M), asym. dimethyl guanidine sulphate (10^{-3}M), aminoguanidine bicarbonate (10^{-4} and 10^{-5}M), caused 100% inhibition of the histaminase activity of cat's kidney extracts, but did not inhibit the histidine decarboxylase activity of rabbit's kidney. Aminoguanidine (10^{-4}M) was therefore chosen as an inhibitor of histaminase activity in further experiments designed to study the distribution of histidine decarboxylase in animal tissues.

The Effect of Organic Solvents on Histidine Decarboxylase

During experiments involving incubation times of 8 hr. or more, one drop (20 mg.) chloroform was added to prevent the growth of bacteria. The added chloroform considerably increased the pro-

TABLE IV

SUMMARY OF THE INHIBITORY ACTION OF VARIOUS SUBSTANCES ON THE HISTIDINE DECARBOXYLASE ACTIVITY OF RABBIT KIDNEY AND ON THE HISTAMINASE ACTIVITY OF CAT KIDNEY

Compound	Molar Concentration	% Inhibition of Histamine Destroying Activity	% Inhibition of Histidine Decarboxylase Activity
Hydrazine sulphate	10^{-3}	100	100
	10^{-4}	100	100
	10^{-5}	100	
	10^{-6}	94·0	
	10^{-7}	69·0	
Girard's reagent "P"	10^{-3}	100	75
	10^{-4}	73·4	37
	10^{-5}	50·2	
	10^{-6}	13·0	
	10^{-7}	0	
Girard's reagent "T"	10^{-3}	83·5	66
	10^{-4}	59·2	21
	10^{-5}	22·0	
	10^{-6}	8·0	
	10^{-7}	0	
B_1 pyrimidine	10^{-3}	96	0
	10^{-4}	76	0
	10^{-5}	50	0
	10^{-6}	20·6	
	10^{-7}	13·5	
Putrescine hydrochloride	10^{-3}	51	0
	10^{-4}	15·7	0
	10^{-5}	5·5	
	10^{-6}	0	
	10^{-7}	0	
Cadaverine dihydrochloride	10^{-3}	31·4	0
	10^{-4}	20	0
	10^{-5}	0	
	10^{-6}	0	
	10^{-7}	0	
Agmatine sulphate	10^{-3}	76	0
	10^{-4}	40·7	0
	10^{-5}	14·9	
	10^{-6}	0	
	10^{-7}	0	
Glyoxaline	10^{-3}	87	0
	10^{-4}	100	0
	10^{-5}	53	
	10^{-6}	29·2	
	10^{-7}	0	
Guanidine carbonate	10^{-3}	76	0
	10^{-4}	33	0
	10^{-5}	9	
	10^{-6}	2	
	10^{-7}	0	
Methylguanidine sulphate	10^{-3}	100	0
	10^{-4}	75	0
	10^{-5}	42	
	10^{-6}	18·1	
	10^{-7}	0	
Asym. dimethylguanidine sulphate	10^{-3}	100	0
	10^{-4}	86	0
	10^{-5}	53	
	10^{-6}	26·4	
	10^{-7}	0	
Aminoguanidine bicarbonate	10^{-3}	100	40
	10^{-4}	100	0
	10^{-5}	100	
	10^{-6}	50	
	10^{-7}	32	
Iproniazid	10^{-3}	49·2	86
	10^{-4}	8·2	17
	10^{-5}	0	
	10^{-6}	0	
	10^{-7}	0	
Chloroform	10^{-3}	Nil (Table VI)	Potentiation 300–400%
Benzene	10^{-3}	Nil (Table VI)	Potentiation 600–1,000%

TABLE V

EFFECT OF CHLOROFORM, BENZENE, TOLUENE AND AMYL ALCOHOL ON THE PRODUCTION OF HISTAMINE FROM HISTIDINE BY RABBIT KIDNEY TISSUE

Each result is the mean of duplicate incubations. Histamine produced expressed as μg. base/g. wet tissue.

	Organic Solvent Added Before Incubation (T')	Organic Solvent Added After Incubation (C'_1)	Organic Solvent Added Before Incubation. No Histidine (C'_2)	Ratio (T'/C'_1)
Chloroform alone				
	19·7	6·7	0·8	2·98
	34·4	10·8	0·5	3·19
	6·8	1·6	0·1	4·2
	11·1	3·2	0·2	3·5
Chloroform and benzene on the same tissue				
Chloroform ..	0·97	0·25	0·01	4·0
Benzene ..	10·0	0·25	0·01	40·0
Chloroform ..	24·9	7·6	0·4	3·5
Benzene ..	56·9	7·6	0·5	7·9
Chloroform ..	36·4	13·5	0·3	2·7
Benzene ..	99·6	13·5	0·2	7·4
Benzene alone				
	114·0	31·2	0·7	3·8
	6·7	1·25	0·3	4·7
	88·6	13·2	0·3	6·7
	15·4	1·2	0·05	12·5
Toluene, benzene and chloroform on the same tissue				
Toluene ..	19·7	2·2	0·1	9·0
Benzene ..	27·6	2·2	0·1	12·5
Chloroform ..	9·0	2·2	0·1	4·1
n-Amylalcohol, benzene and chloroform on the same tissue				
n-Amyl alcohol ..	15·0	13·6	0·2	1·1
Chloroform ..	36·4	13·5	0·2	2·7
Benzene ..	99·6	13·5	0·4	7·4

duction of histamine. A series of experiments was therefore carried out, in which one drop (approximately 20 mg.) of chloroform, of benzene, of toluene, or of amyl alcohol, replaced the 1 ml. aqueous solution of the test substance.

The results, summarized in Table V, show that chloroform, benzene and toluene potentiate histidine decarboxylase, and that the effect is on the *formation* of histamine, and is not due to *release* of histamine. Benzene increased the production of histamine some 7-fold within the limits 4 to 40; whereas chloroform increased the production 3- to 4-fold. Toluene was almost as active as benzene, whilst amyl alcohol had no significant action. It was therefore decided to use benzene to increase histamine production in all experiments involving histidine decarboxylase activity.

Some experiments were next carried out to determine the nature of the benzene effect, which was shown to a varying degree by a large number of organic solvents. The results will form the subject of another paper.

Inhibition of the Histaminase Activity of an Acetone-dried, Purified Powder of Hog's Kidney

Since there is evidence that more than one enzyme in tissues may destroy histamine (Kapeller-Adler, 1949; Zeller *et al.*, 1953; Schayer, 1953a, b), the most active substances in Table III were also tested for their action on an acetone-dried purified powder from hog's kidney. The results (Table VI) were similar to those obtained with cat's kidney (Table III) and do not therefore suggest any difference between the enzymes obtained from the kidneys of cats and of hogs.

Distribution of Histidine Decarboxylase, Histaminase, and Histamine in the Tissues of Various Species

Having obtained a substance which would potentiate histidine decarboxylase (benzene) and another which would inhibit histaminase (aminoguanidine) it was felt that a more accurate knowledge of histidine decarboxylase distribution could be obtained by using benzene and aminoguanidine in experiments designed to show the histidine decarboxylase activity of various tissues.

The results given in Table VII, and summarized in Table VIII, have been obtained from duplicate experiments on separate animals or groups of animals. The results for histidine decarboxylase activity refer to the histamine formed per g. tissue

TABLE VI

INHIBITION OF THE HISTAMINASE ACTIVITY OF HOG'S KIDNEY

Each result is the mean of duplicate incubations. The figures in parentheses are the corresponding results for the test against the histamine-destroying activity of the cat's kidney.

Test Substance	Final Molar Concentration	μg. Histamine Remaining after Incubation			% Inhibition of Histaminase Activity
		With Test T'	Without Test C'_1	Histamine Blank C'_2	$\frac{T'-C'_1}{C'_2-C'_1}\times 100$
Putrescine hydrochloride*	10^{-2}	10·3	5·4	10·1	100
	10^{-3}	7·8	5·1	9·9	56 (51)
Cadaverine dihydrochloride*	10^{-2}	9·9	5·4	10·1	100
	10^{-3}	7·2	5·1	9·9	44 (31·4)
Agmatine sulphate*	10^{-2}	10·5	5·4	10·1	100
	10^{-3}	8·9	5·1	9·9	80 (76)
Aminoguanidine bicarbonate†	10^{-4}	19·1	10·2	19·3	100 (100)
Guanidine carbonate†	10^{-4}	14·9	10·2	19·3	52 (32)
Asym. dimethylguanidine sulphate†	10^{-4}	16·1	10·2	19·3	65 (86)
Iproniazid†	10^{-3}	14·4	10·2	19·3	46 (49·2)
Histidine monohydrochloride*	$1{\cdot}5\times10^{-3}$	4·4	4·4	10·2	0
Benzene*	2×10^{-2}	4·9	4·6	10·2	0
Benzene†	2×10^{-2}	9·1	10·2	19·3	0
Chloroform*	2×10^{-3}	4·7	4·9	10·1	0
Chloroform†	2×10^{-3}	9·3	9·6	19·3	0

* 10 μg. histamine added to incubation mixture.
† 20 μg. histamine added to incubation mixture.

TABLE VII

TISSUE DISTRIBUTION OF HISTIDINE DECARBOXYLASE, HISTAMINASE AND HISTAMINE

Each result is the mean of duplicate incubations. For each tissue the results of experiments on 2 animals are given. Values in each of the 3 parts of the Table are from the same 2 animals of each species.

	Kidney		Liver		Whole Stomach		Duodenum		Ileum		Colon		Lung		Pancreas	
Histidine decarboxylase activity (μg. histamine formed/g. tissue)																
Man *	0·2	1·1	0	1·8	0·1	0	0	0	0	0·6						
Dog	0	0	0	0	0	0·7	0	0	0	0			0	0		
Cat	0	0	0	0	0	0	0	0	0	0			0	0	0	0
Rabbit	114	23	17	15	2	14	0	1·1	1·9	7	0	0	0	0	0	0
Guinea-pig	81	27	25	12	6	5	77	15	31	20	1·5	1·1	0·6			
Rat	4	6	0·6	6	4	5	3	3	0	0	0·9	0	0			
Mouse	287	67	5	7	5	12	1	4	0	0	0	0	0			
Histaminase activity (μg. histamine destroyed/g. tissue)																
Man *	21	20	1·5	0	1·4	0	26	14	26	35						
Dog	>170	>250	12	4	0	0	30	35					6	3		
Cat	>170	>250	0	1	4	0·5	70	36	57	37			3	4	5	2
Rabbit	0	0	0	0·4	0	0	2	11	1·0	11	1·3	1·2	3	4	1·9	1·8
Guinea-pig	0·9	0	6	0	0·1	0·1	5	9	6	13	0·6	0·4	1·4			
Rat	1·1	0	0·8	0	7	0	10	26	15	32	10	4	0·6			
Mouse	0	0	0	0	0·9	0	4	20	20	0	4	2	1·2			
Histamine present (μg. histamine present/g. tissue)																
Man *	1	2	0·8	3	10	48	16	65	24	57						
Dog	0	0	14	8	42	24	30	37	25	33			84	61		
Cat	0	0	0	0	16	10	40	31	36	15			29	9	1·0	0
Rabbit	0·5	0·1	1·3	0·5	6	3	6	14	1·5	1·6	3	4	18	7	0	0
Guinea-pig	1·8	0·2	3	0·3	3	10	6	17	5	13	2	3	11			
Rat	0	0	1	0	37	29	5	16	11	26	9	4	16			
Mouse	4	0	0	0	5	10	1·5	0	1·1	0	0	0	0			

* 6 hr. *post-mortem* tissue. First died of acute bronchitis, age 49 years; 2nd died cardiac failure following prostatectomy, age 82 years.

TABLE VIII

THE HISTAMINE CONTENT AND DISTRIBUTION OF HISTIDINE DECARBOXYLASE AND HISTAMINASE ACTIVITIES OF ANIMAL TISSUES

The symbols have the following meanings:

−=	<2 μg./g. tissue	
+=	2— 10	„ „
++=	10— 30	„ „
+++=	30—100	„ „
++++=	100—250	„ „
+++++=	>250	„ „

	Man	Dog	Cat	Rabbit	Guinea-pig	Rat	Mouse
Histidine decarboxylase (histamine formed)							
Kidney	−	−	−	+++	+++	+	++++
Liver	−	−	−	++	++	−	+
Stomach	−	−	−	+	+	++	++
Duodenum	−	−	−	−	+++	+	+
Ileum	−	−	−	+	++	−	−
Colon				−	−	−	−
Lung		−	+	−	−	−	−
Skin, voluntary muscle, spleen and caecum. Nil.							
Histaminase (histamine destroyed)							
Kidney	+++	+++++	+++++	−	−	−	−
Liver	−	+	−	−	−	−	−
Stomach	−		−	−	−	−	−
Duodenum	++	+++	+++	++	−	++	+
Ileum	+++	+++	+++	+	+	+++	++
Colon				+	−	+	+
Lung		−	+	+	+	−	−
Skin, voluntary muscle, spleen and caecum. Nil							
Histamine (histamine naturally present)							
Kidney	−	−	−	−	−	−	−
Liver	+	+	−	−	−	−	−
Stomach	+++	−	++	++	+	++++	+
Duodenum	+++	+++	+++	++	++	+	−
Ileum	+++	+++	+++	+	+	++	−
Colon				+	+	+	−
Lung		+++	+++	++	++	++	−
In rabbit: spleen +++; caecum ++; skin, voluntary muscle, nil.							

after 3 hours' incubation. The results for histaminase activity are in terms of the amount of histamine destroyed per g. tissue after 3 hours; histamine content is per g. tissue before incubation.

The value of aminoguanidine as a histaminase inhibitor in the histidine decarboxylase experiments is evident; in nearly all the experiments in which histaminase, but not histidine decarboxylase, was present, no histamine was destroyed even after 3 hours' incubation.

Intracellular Distribution of Histidine Decarboxylase, Histaminase, and Histamine

High speed differential centrifugation was tried as a method to separate histidine decarboxylase from histaminase. This might be successful if the enzymes were contained in different particulate cell bodies. The intracellular distribution of histamine was also determined.

TABLE IX

HISTAMINE FORMED BY RABBIT KIDNEY TISSUE EXTRACTS SUBJECTED TO VARYING GRAVITATIONAL FORCES AND BY THE RESIDUES (*italic numerals*) FROM EACH CENTRIFUGATION

The results are expressed in μg. histamine formed/g. original tissue. Each result is the mean of duplicate incubations.

Original Minced Tissue	1st Centrifugation 2,500 *g*	2nd Centrifugation 37,000 *g*	3rd Centrifugation 90,000 *g*	Presence of Benzene
7·0	6·4	6·0	6·2	–
	0·8	*0·9*	*0·2*	
31·3	27·6	37·9	31·2	+
	5·2	*2·1*	*0·4*	
3·1	4·8	3·2	3·5	–
	0	*not done*	*0·2*	
17·5	16·5	not done	16·2	+
	1·3	*0·2*	*0·3*	

Histidine decarboxylase in rabbit kidney tissue (Table IX) and histaminase in rabbit lung tissue (Table X) are either contained in the cell sap, or pass readily into it, since cell free tissue extract subjected to gravitational forces of 90,000 *g* and 37,000 *g* were as active as minced tissue. Of the histamine contained in the rabbit's lung, 66% was present in the "mitochondrial" fraction, which contains most cell granules, while the rest was present in the supernatant fluid after centrifugation at 37,000 *g* for 1 hour. The histamine may have been liberated *post mortem* or may have been originally present in the cell sap. The results are summarized in Table XI.

TABLE X

HISTAMINE DESTROYED BY RABBIT LUNG TISSUE EXTRACTS SUBJECTED TO VARYING GRAVITATIONAL FORCES, AND BY THE RESIDUES (*italic numerals*) FROM EACH CENTRIFUGATION

The results are expressed as μg. histamine destroyed/g. original tissue/1 hr. Each result is the mean of duplicate incubations.

Original Minced Tissue	1st Centrifugation 2,500 *g*	2nd Centrifugation 37,000 *g*
3·2	3·4	2·7
	0	*0*
5·8	6·0	3·5
	0	*0*

TABLE XI

THE INTRACELLULAR DISTRIBUTIONS OF HISTIDINE DECARBOXYLASE IN RABBIT KIDNEY TISSUE AND OF HISTAMINASE AND HISTAMINE IN RABBIT LUNG TISSUE

The limits of several estimations are given in parentheses.

Cell Fraction	Histidine Decarboxylase Activity	Histaminase Activity	Histamine
Cell debris ..	5·8% (0–16·6)	nil (<0·2%)	6·3% (2·9–9·7)
Mitochondria ..	2·1% (0–12·8)	nil (<0·2%)	76·9% (60·2–93·6)
Microsomes ..	0·4% (0–1·4)	72·4% (60·4–84·4)	24·3% (19·4–29·3)
Supernatant fluid	95·8% (52–150)		

DISCUSSION

The present work confirms the results obtained by Werle and Holtz and their respective co-workers, and provides additional evidence of the occurrence in certain animal tissues of an enzyme capable of decarboxylating histidine and forming histamine. The method used is believed to be more accurate than that used by earlier investigators, who either partially removed histaminase by shaking with kaolin, or failed to remove or inhibit it.

Histidine decarboxylase is present in the kidney, liver, stomach and duodenum of the rabbit, guinea-pig, mouse and rat, and in the ileum of the rabbit and guinea-pig. It is absent from the colon, caecum, pancreas, spleen, lung, skin and skeletal muscle of these species and is also absent from the corresponding tissues of the cat, dog, and man. In rodents, histaminase and histamine appear to be present together in the duodenum, ileum, lung and caecum and sometimes in the colon. Of these tissues, only the duodenum of each animal and the ileum of rabbits and guinea-pigs contained any histidine decarboxylase. The stomach in every species tested contained large quantities of histamine and some histidine decarboxylase, but no detectable histaminase. In cat, dog, and man, histidine decarboxylase was not found, but there was high histaminase activity in the kidney, duodenum, and ileum. Small amounts of histaminase were found in cat and dog lung and pancreas. No histamine was found in the kidneys of rodents, though the kidneys appear to be the chief histamine-forming organs in these species; histidine decarboxylase was absent from many tissues which contain histamine, especially those of

man, dog, and cat. This suggests that, if tissue histidine decarboxylase is the source of histamine in the body, then histamine must be transported to and stored by these tissues.

The enzyme appears to be in the cell sap, since a tissue extract subjected to a gravitational force of 90,000 *g* had an enzyme activity comparable to that of minced tissue (Table IX). This tissue extract would contain no particulate cell components. The enzyme appears to pass readily from the cells into the surrounding vehicle and its presence in any particulate fraction could not be demonstrated. About 66% of the total histamine found in rabbit lung tissue was present in the mitochondrial fraction. The remainder was found in the supernatant fluid after centrifugation at 37,000 *g*; this may have been liberated *post mortem*. This result agrees with the work of Copenhaver, Nagler, and Goth (1953), confirmed by Hagen (1954), which showed that most of the histamine in the dog liver is in the mitochondrial fraction.

The distribution of histidine decarboxylase (Table VII) in the cat, dog, rabbit, guinea-pig, mouse, and rat differs in some respects from that shown by Werle (1943), who records the presence of histidine decarboxylase in the kidney and liver of the cat. Werle's data are based on the work of Holtz and Heise (1937b) and Holtz, Heise, and Spreyer (1938), who incubated the tissues in the presence and absence of histidine and tested for histamine content on the cat's blood pressure. Their test and control samples differed in one important respect—one did not contain histidine. The authors do not seem to have excluded the possibility that the small effect which they observed may have been due to histamine present as an impurity in the sample of histidine used.

The presence of histidine decarboxylase in the guinea-pig has been conclusively demonstrated in several tissues by Werle and his co-workers, by Holtz and his co-workers, by Schayer (1952), and by me. It is therefore quite possible that systemic histidine is converted to histamine in the guinea-pig. It does not follow that histamine is formed from histidine in the tissues of all mammals; and, even in rodents, a local decarboxylation of histidine is not necessarily the only source of histamine.

Wilson (1954) demonstrated that inhibition of the intestinal bacterial flora of rats by orally administered antibiotics or sulphonamides causes a decrease in the urinary excretion of histamine. These experiments seem to support an alternative theory—namely, that the histamine in mammalian tissues originates from histamine absorbed from the intestine, where it is formed as a result of bacterial activity. The presence of histaminase in the intestinal wall has been advanced as evidence against this theory. However, some of the histamine formed in the intestine by bacteria may be combined with protein or other molecules (Rocha e Silva, 1943; Rocha e Silva and Andrade, 1943) and may be absorbed in the form of a complex compound, in a pharmacologically inactive but easily convertible form, which is not attacked by histaminase. Histaminase may prevent the entry only of free, pharmacologically active, histamine.

The present work supports the view that the histamine in the body tissues has been absorbed from the intestine; in man, cats, and dogs, the presence of histidine decarboxylase has not been demonstrated—contrary to what would be expected if histamine were formed from histidine in the tissues of these species. Sufficient histamine may be absorbed from the gut in carnivorous animals to meet all physiological needs, so that no endogenous formation of histamine is necessary and, consequently, a histamine-forming enzyme is not required. In rodents, insufficient histamine may be absorbed from exogenous sources, and, to make good the deficit, histamine may be formed from histidine by the action of histidine decarboxylase in the kidney, liver and duodenum. It is possible that histidine decarboxylase is only present in the tissues of those animals which do not obtain sufficient histamine from their gut contents.

Summary

1. The formation of histamine in the mammalian body, from systemic histidine, by a tissue enzyme, histidine decarboxylase, has been reinvestigated. Proof of the presence of such an enzyme in the kidneys, liver and duodenal tissues of certain rodents has been presented. No histidine decarboxylase was found in tissues from man, dogs and cats.

2. Certain organic solvents powerfully potentiate histidine decarboxylase activity. The inhibition of histaminase by aminoguanidine (10^{-4}M) has been shown to be specific.

3. The distribution of histidine decarboxylase in several tissues of man, dog, cat, rabbit, guinea-pig, rat, and mouse has been studied, using benzene to potentiate histidine decarboxylase activity and aminoguanidine to inhibit histaminase activity. At the same time estimates were made of the histaminase and histamine contents of the tissues.

4. Studies of the intracellular distribution of histidine decarboxylase, histaminase, and histamine showed that histidine decarboxylase and histaminase are present in the cell sap. Two-thirds of the

histamine is in the mitochondrial fraction; the remainder is in the cell sap.

5. Intracellular decarboxylation of histidine is probably not the major pathway in the formation of histamine in carnivorous animals, and may only be a secondary route in rodents.

I express my thanks to Professor J. H. Gaddum, F.R.S., for his help and guidance in this study; to Dr. R. Kapeller-Adler for a sample of acetone-dried, purified, hog's kidney; and to Dr. R. MacFarlane, Pathology Department, Western Infirmary, Glasgow, for the *post mortem* human tissues. I also acknowledge the award of a Medical Research Council grant, the tenure of which made this work possible.

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Brit. J. Pharmacol. (1956), **11**, 128.

PROTECTION BY CYSTEINE AGAINST THE ACUTE TOXICITY OF A CHEMICAL RADIO-SENSITIZER ("SYNKAVIT")

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(RECEIVED SEPTEMBER 21, 1955)

The vitamin K substitute tetrasodium 2-methyl-1 : 4-naphthohydroquinone diphosphate ("Synkavit") has been shown to act as a radio-sensitizer in tissue culture (Mitchell and Simon-Reuss, 1947, 1952) and in the radiation treatment of certain tumours in rats and in human patients (Mitchell, 1953a, 1955). Mitchell (1953b) suggested that the radiosensitization might be due to an interaction with thiol groups.

Synkavit, injected intravenously, lowers oxidation-reduction potentials in rat muscle and tumours (Cater and Phillips, 1954) and in all other tissues that have been tried (Cater and Phillips; Silver, Cater, and Phillips; in preparation). Several drugs that protect against radiation, including cysteine, cause a fall in these potentials in tumour, muscle, and lactating mammary gland, but cysteine causes a rise in cerebral grey matter. These observations led us to inquire whether cysteine would potentiate or antagonize the acute toxic effects of synkavit.

METHODS

White Wistar rats weighing between 140 and 230 g. were used. Males and females were kept separately and were used in different experiments. In any one experiment, rats of one sex were weighed and then allocated to experimental groups so that the rats of each group had approximately the same distribution of weights. Care was taken to avoid any bias in this process, which was preferable to strict randomization because of the wide range of weights and the small numbers in the groups. After allocation to their groups, rats were kept singly in individual boxes, without food or water, until about 4 hours after the last injection; they were then returned to cages, in their groups, with free access to food and water.

In every experiment but one, cysteine was injected first. It was weighed as hydrochloride, dissolved in boiled distilled water, and (in most experiments) partly neutralized to *p*H 3–6 with boiled N-NaOH immediately before injection. Under these conditions there was no visible precipitation of cystine for many minutes. The total volume injected was 0.6–1.0 ml. Unprotected groups were injected with a similar volume of isotonic saline. Injections were made into the lateral tail vein after 1 min. immersion in warm water. After the required interval, tetrasodium 2-methyl-1 : 4-naphthohydroquinone diphosphate ("Synkavit" Roche, 0.3 M) was injected into the opposite tail vein. Dosage of each drug was proportional to body weight.

The time of survival after the synkavit injection was recorded. Nearly all the rats that were injected with synkavit alone either died within 45 minutes or survived for at least several hours. The first assessment of the effect of an injection sequence was made on the proportion of deaths within 45 minutes. Results assessed in this way are presented in Table I. An assessment was also made on the basis of the mean reciprocal survival time for each group of rats. This particular transformation was chosen to allow the inclusion of individuals that survived indefinitely (Gaddum, 1953), and it proved to be the most convenient assessment for much of the data (Fig. 1).

RESULTS

For the first series of experiments a dose of synkavit, 1.3 mM/kg., was chosen to give 100% mortality within 45 min. Marked protection was obtained when an equimolecular proportion of cysteine hydrochloride was injected 3 min. before or 3 min. after the synkavit. There was still protection when the same dose of cysteine was administered up to an hour before the synkavit. Three times the dose of cysteine, neutralized, gave protection at 1 hour and at 3 hours, and also apparently at 24 hours, although subsequent experiments with the same and different doses of synkavit failed to confirm any protection at 24 hours.

In the second series of experiments the same dose of synkavit was used; but the rats had a higher susceptibility to the drug. Very marked protection

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was still found with the larger dose of cysteine given one hour before the synkavit.

The results for these two series are summarized in Table I.

In the third and longest series of experiments, the susceptibility to synkavit (alone) remained fairly constant. The LD50, for death within 45 minutes, was approximately 60 mg. (free ester)/200 g. rat (0.9 mM./kg.). Several doses of synkavit were used, with small groups of rats, while the protected groups had the same or larger doses of synkavit; the dose of cysteine (partly neutralized) was always 3.9 mM./kg., given one hour before the synkavit. Very marked increase in survival times was found, with no deaths within 45 minutes even at doses of synkavit over twice the LD50. The results of this series are summarized graphically in Fig. 1.

A similar experiment was carried out using glutathione at a dose of 1.6 mM./kg. The dotted lines in Fig. 1 refer to this experiment, and show an increase in survival times for the protected groups. A single group, matched with the same control

TABLE I

PROTECTION BY CYSTEINE AGAINST A LETHAL DOSE OF SYNKAVIT (1·3 mM./kg.)

Cysteine		No. of Rats	Deaths Within 45 min.	Alive and Well at 48 hr.	*P*
mM./kg.	Time				
First series					
0·0		27	27	0	—
1·3	3 min. before	8	1	6	0·000001
1·3	3 ,, after	5	0	4	0·000005
1·3	½ hr. before	6	2	1	0·0004
1·3	1 ,, ,,	5	2	3	0·0020
3·9	1 ,, ,,	5	0	0	0·0004
3·9	3 ,, ,,	5	2	0	0·0020
3·9	24 ,, ,, (i.p.)	8	5	2	0·0036
Second series					
0·0		8	8	0	—
1·3	1 hr. before	9	8	0	—
1·3	3 ,, ,,	5	5	0	—
3·9	1 ,, ,,	7	0	1	0·0002
3·9	3 ,, ,,	6	6	0	—
Control: No synkavit					
4·7 mM./kg. cysteine, followed by saline		6	0	6	—

P, the probability of getting a particular result by chance, has been calculated by the exact method for 2 × 2 tables with fixed marginal totals (Fisher, 1946). Each protected group has been compared with the unprotected group of the same series, taking account of deaths within 45 minutes only.

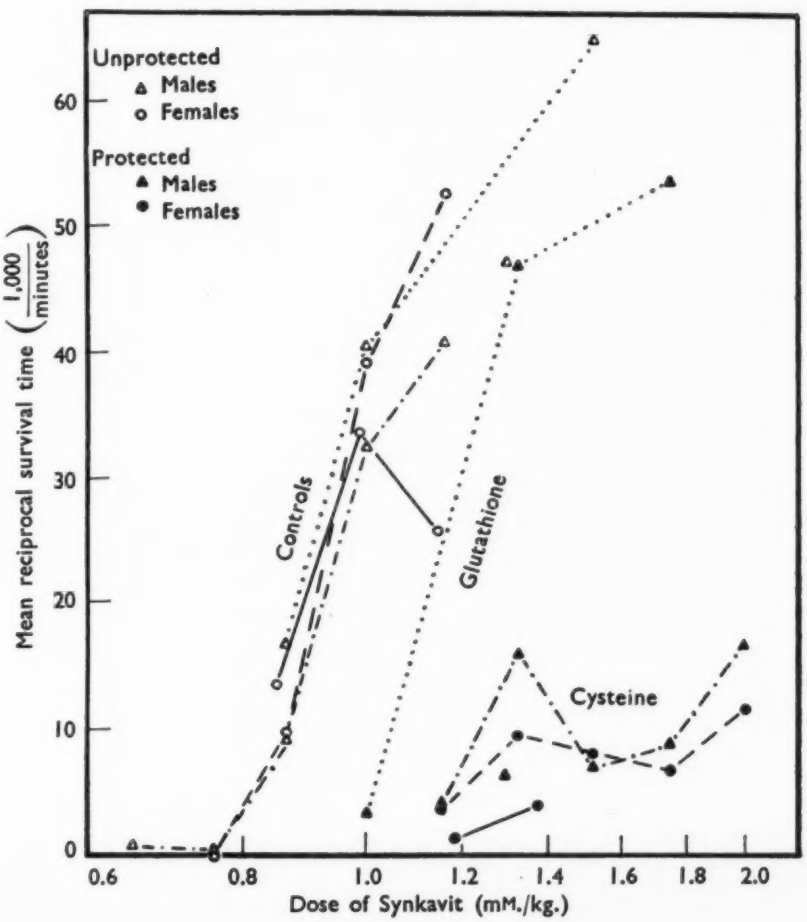

FIG. 1.—Effect upon reciprocal survival time of different doses of "Synkavit" administered alone, and one hour after protection with either cysteine (3·9 mM./kg.) or glutathione (1·6 mM./kg.). Each point represents the mean value for a group of rats (average 6 rats per group). Each set of open symbols joined by a line, together with the corresponding set of solid symbols, represents one experiment with groups matched for weight distribution, and used on the same day. (In one of the experiments there was only one protected and one unprotected group, which are represented by two isolated points.)

groups, received 3.3 mM./kg. of glutathione followed by synkavit, 1.3 mM./kg. The survival time was further increased, but was shorter than with cysteine at a dose of 3.9 mM./kg. Owing to the high cost of pure glutathione, the groups in this experiment were only of 3 rats each.

A control group of rats received neutralized cysteine alone, 4.7 mM./kg.; and another small group received the synkavit solvent alone (kindly supplied by Dr. A. L. Morrison of Roche Products, Ltd.), in amount corresponding to twice the largest dose of synkavit. Neither of these groups showed more than a transient malaise, and both appeared perfectly well before they were killed. Careful naked-eye post-mortem examination revealed no abnormality.

No differences attributable to sex were found either with synkavit alone or with cysteine plus synkavit. No trend of mortality or survival time with body weight was apparent for protected or unprotected rats, showing that the method of adjusting dose to body weight by simple proportion was satisfactory. Further analysis of the pooled " unprotected " results showed that this adjustment was more satisfactory than one depending on the 2/3 power of body weight or any lower power.

It was noted that unprotected animals frequently became very excitable shortly after the injection of a lethal dose of synkavit, and tended to leap against the sides of their cage. This excitable stage might proceed to convulsions before the animal died. In the protected animals the tendency to convulsions occurred later; the convulsions might start an hour after injection and last for 2 or 3 hours, before either death or recovery. Animals dying after a prolonged series of convulsions went into rigor mortis immediately.

We investigated the possibility that the convulsions might have been due to hypoglycaemia, and found that this was not so. Samples of blood (0.2 ml.) were taken from the tail veins of 5 rats, and kept for blood sugar estimation. Synkavit, 0.9 mM./kg., was then injected. After twenty minutes, 4 of the rats showed convulsions either spontaneously or in response to a sharp sound, and a second blood sample was taken from each by cardiac puncture. The blood sugar levels before injection ranged from 82–102 mg./100 ml.; and 20 minutes after injection they ranged from 131 to 199 mg./100 ml. The fifth rat, which had no convulsion, showed a rise of blood sugar from 92 to 284 mg./100 ml. in the 36 minutes after injection. Control estimations on normal rat blood before and after the addition of known amounts of synkavit, and of unphosphorylated 2-methyl-1: 4-naphthohydroquinone, showed that both substances, particularly the latter, could raise the reading obtained. The effect was not sufficient, however, to account for the whole of the observed rise after injection. There was, therefore, no fall of blood sugar to account for the convulsions.

Animals that died some hours after injection of synkavit, whether " protected " or not, frequently passed blood-stained urine, and post-mortem examination showed intense congestion of the kidneys. Sections of the kidneys showed great engorgement of the vessels and glomerular capillaries, and the presence of blood and protein exudates in the lumen of the tubules.

Discussion

Our results appear to be consistent with Mitchell's suggestion (1953b) that synkavit reacts with thiol groups in enzyme molecules. Friedmann (1954) showed that both cysteine and glutathione form addition products with synkavit, but we have no information on the toxicity of such compounds. Protection at as long an interval as 3 hours, with the relative molecular proportions used, would be difficult to explain on the basis of chemical neutralization of the synkavit in the blood.

The tendency of synkavit to cause convulsions suggests that the brain may be the organ most vulnerable to its toxic action. In this connexion it may be significant that we observed in brain a rise of oxidation-reduction potential after cysteine, and a fall after synkavit; whereas in other tissues both these drugs cause a fall.

In our unprotected groups, the LD50 for intravenous injection of synkavit (counting only deaths within 45 minutes) was 300 mg./kg. (expressed in accordance with the manufacturer's practice as free ester, mol. wt. 334). This may be compared with the LD50 found by Smith, Ivy, and Foster (1943) for rats, by subcutaneous injection, of 385 mg./kg. None of these rats died in less than 12 hours, and the average time of death was 30 hours. Foster (1940) also found LD50's of 280 mg./kg. for mice both by intravenous and by subcutaneous injection, and 425 mg./kg. for chicks by subcutaneous injection. (These values have been recalculated by us in terms of the free ester; in the works cited they were given in terms of the hydrated tetrasodium salt.) Smith *et al.* observed no excitability or convulsions in rats, but Foster records that a rabbit, injected intravenously with 200 mg./kg. of the tetrasodium salt (125 mg./kg. free ester), became excited, had convulsions at 30 minutes, and died 35 minutes after injection.

It is interesting that cysteine can protect against the toxic effects of nitrogen mustard (Nadkari,

Goldenthal, and Smith, 1954; Therkelsen, 1956), which has a radiomimetic action. We do not, however, regard synkavit as a radiomimetic drug, and we are not clear what relationship, if any, there is between these results and our own.

Summary

1. Tetrasodium 2-methyl-1 : 4-naphthohydroquinone diphosphate ("Synkavit") injected intravenously into rats had an immediate toxic action which caused death within 45 minutes. The LD50 was approximately 0.9 mM./kg. Rats which survived beyond 45 minutes usually lived for many hours or indefinitely.

2. Cysteine, injected at the same time or up to three hours before, in doses of 1.3 and 3.9 mM./kg., protected a high proportion of rats against the immediate toxic effect of synkavit, and increased the average survival times after doses of synkavit ranging from 1.0 mM./kg. to 2.0 mM./kg.

3. Cysteine was not itself toxic in doses up to 4.7 mM./kg.

4. Glutathione exerted a similar protective effect. The increase in survival time was comparable to that obtained with cysteine at the same molar dose.

5. Synkavit in toxic doses caused hyperexcitability and convulsions, which were not due to hypoglycaemia. This suggests an action on the central nervous system; oxidation-reduction potential measurements give some support to this view.

We wish to thank Professor J. S. Mitchell for his interest and advice, Professor J. H. Gaddum for advice in planning part of the work, and Dr. D. N. Lawrie for the estimations of blood sugar. Miss N. Burr looked after the animals and gave very competent assistance during the experiments.

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THE ROLE OF BLOOD-PRESSURE LEVEL IN THE PRODUCTION OF VENTRICULAR ARRHYTHMIA IN THE RABBIT

BY

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(RECEIVED OCTOBER 6, 1955)

During a study of the pressor effects of noradrenaline in normal and in hypertensive rabbits (Conway, 1955) it was found that the response to progressively increasing doses of noradrenaline by intravenous infusion was limited quite suddenly by the emergence of a characteristic cardiac irregularity (Fig. 1). This was thought to be due to a direct effect of noradrenaline on the myocardium, as had been demonstrated for adrenaline (Levy, 1913, 1914; Allen, 1934), and latterly for noradrenaline (Meek, 1941). Further study, however, showed that this was unlikely to be so, since the emergence of the arrhythmia was independent of the dose used. It was thought therefore that in the rabbit, at least, ventricular arrhythmia could be produced in circumstances in which the direct cardiac effects of adrenaline or noradrenaline could only be of secondary importance, and that the level of the blood pressure might be the essential factor.

METHODS

Nineteen normal and four hypertensive rabbits have been studied; of the hypertensive rabbits, three had been studied in their normal state and are therefore represented in both groups. Ten of the normal rabbits and the four hypertensives were studied in the conscious state and in six animals the experiments were repeated on more than one occasion. Hypertension was produced by enclosing the kidney in a latex capsule (Flasher, Drury, and Sobin, 1949). To measure the response to pressor drugs the conscious rabbits were placed in a heated box, where they remained, without restraint, until the ear artery was well dilated. After infiltration of the nerve at the base of the ear with 2% lignocaine, a needle (27 SWG) was lodged in the central artery and connected by a saline-filled polythene tube (approx. 20 cm.) to a condenser manometer and records were made with direct-writing oscillographs. The frequency response of the entire system was 30 c./sec. The diastolic blood pressure has been quoted throughout these experiments. Infusions or injections were given into the marginal vein of the same ear, and at each experiment the conditions required to produce the arrhythmia were usually produced three times.

In preliminary experiments, hexamethonium had been found to assist the production of arrhythmia by permitting a greater and more rapid rise in pressure to occur for a given dose of pressor drug without the complication of vagal slowing. In four experiments the effect of vagal section upon the production of arrhythmia has been shown. All but these four experiments were performed, therefore, after full doses of hexamethonium had been given. The initial dose of hexamethonium varied from 40 to 60 mg., with further doses of 10 mg., the criteria for which will be discussed later.

Electrocardiographic studies could not be made in conscious rabbits. Studies were therefore made in 9 rabbits under anaesthesia with pentobarbitone sodium (30 mg./kg.). Blood pressure measurements were taken from the femoral, carotid, or central artery of the ear, and an electrocardiogram was made from bipolar needle electrodes (from two fore-limbs or from one fore-limb and the chest).

Five of the normal animals and the four hypertensives were studied in the conscious state with infusions of

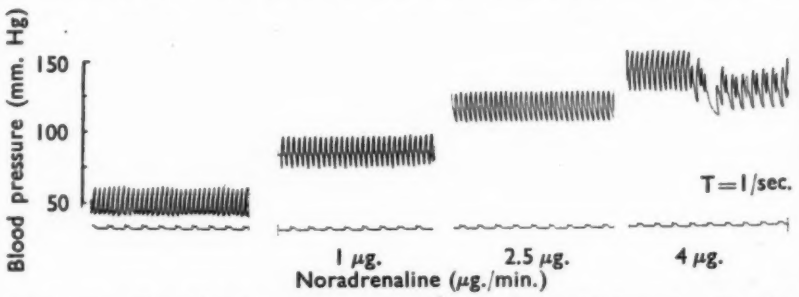

FIG. 1.—The effect of infusions of progressively increasing doses of (−)-noradrenaline after hexamethonium in a conscious rabbit.

noradrenaline alone. Increasing doses were used to produce step-wise increments of pressure until the abnormality appeared. (−)-Noradrenaline bitartrate in saline was used and doses quoted are of the base. For the remaining conscious animals, and those studied under anaesthesia, the effects of infusions of posterior pituitary extract and injections of renin were used in addition to noradrenaline either alone or in combination with the pituitary extract or renin. The posterior pituitary extract was the commercial product "Pitressin" (Parke Davis), and the renin was a lyophilized extract of hog renin kindly given by Dr. H. Goldblatt.

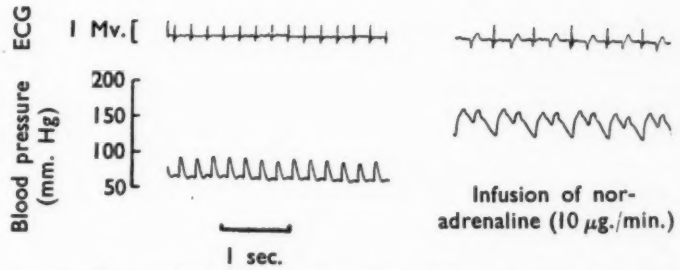

FIG. 2.—The production of ventricular arrhythmia by an infusion of (−)-noradrenaline in an anaesthetized rabbit after hexamethonium.

RESULTS

The Nature of the Cardiac Irregularity

When noradrenaline was administered by intravenous infusion the pressor response was limited, quite suddenly, by the emergence of a characteristic arrhythmia (Fig. 1). The electrocardiographic characteristics of the arrhythmia were observed therefore in the 9 experiments conducted under general anaesthesia. The effect of the arrhythmia on the blood pressure was characteristic. As the blood pressure rose the cardiac arrhythmia appeared suddenly as a premature ventricular beat occurring regularly in alternate heart beats (Fig. 2). Sometimes the premature beat was preceded by a P wave occurring normally in the cardiac cycle with consequent shortening of the P–R interval (Figs. 3 and 4). The resultant pulse wave characteristically showed bigeminal rhythm with the premature beat weaker than the normal one. When the prematurity of the ventricular beat was very slight, pulsus alternans appeared (Fig. 4). The ventricular beats arose initially from a single focus; if the abnormality was allowed to persist it became more severe, ventricular tachycardia appeared (Fig. 3) and in 4 experiments bizarre patterns arising from multiple ventricular foci appeared. In some experiments elevation of the blood pressure was accompanied by depression of the S–T segment of the electrocardiogram (Fig. 5). The importance of this change could not be ascertained, but it was not regarded as an essential feature since the ventricular arrhythmia was produced in the absence of S–T depression.

The mean diastolic pressure in the conscious rabbits at rest, measured from the dilated ear artery, ranged from 50–68 mm. Hg; this was approximately 10 mm. Hg below the aortic or carotid blood pressure. Infusions of (−)-noradrenaline (4–5 μg./min.) in the normal rabbits, after large doses of hexamethonium, produced the ventricular arrhythmia at an average blood pressure of 115 mm. Hg (Table 1A). This did not appear to distress the animals in any way. Large doses of hexamethonium were used in these experiments because the arrhythmia was produced more readily in the absence of the full action of the depressor reflexes. The adequacy of the ganglionic blockade was judged by production of maximal depression of blood pressure, fixation of the pupillary light reflex, and the absence of further potentiation of the pressor response to noradrenaline or change in heart rate.

Vagal activity was not responsible for the cardiac

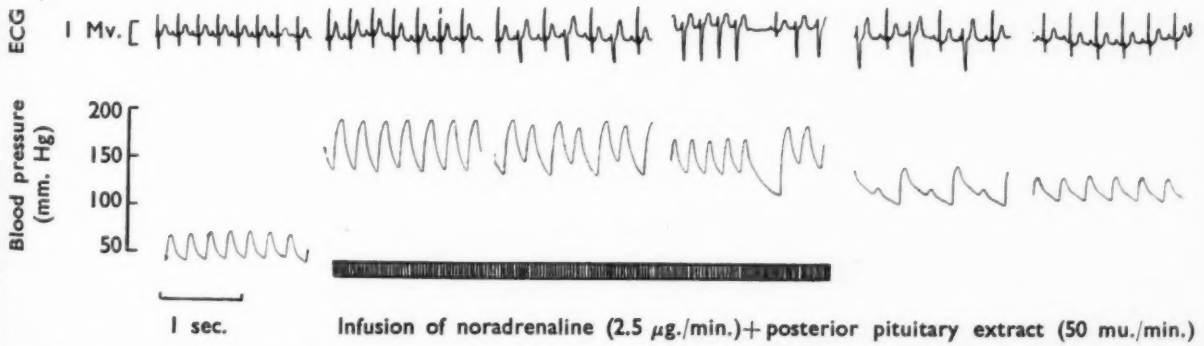

FIG. 3.—The production of ventricular arrhythmia by a mixture of (−)-noradrenaline and posterior pituitary extract, showing the increasing severity of the arrhythmia with a prolonged infusion. Sections of tracing shown were taken at one minute intervals.

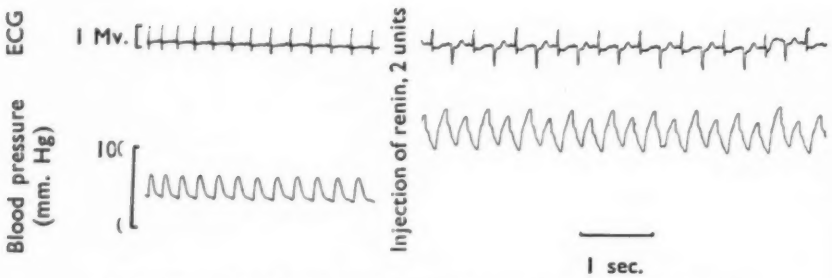

FIG. 4.—The production of ventricular arrhythmia by an injection of renin.

TABLE I

THE PRODUCTION OF VENTRICULAR ARRHYTHMIA BY INFUSIONS OF NORADRENALINE (4–5 μG.) IN NORMAL AND IN HYPERTENSIVE RABBITS

Rabbit No.	Average Pressure at which Arrhythmia Appeared (mm. Hg)
A. *Normal rabbits*	
99 (4 occasions)	124
89 (2 ,,)	116
78	112
88 (2 ,,)	108
79	116
	Mean 115 (S.D. ±6)
B. *Hypertensive rabbits*	
78 (2 occasions)	200
71	174
88 (2 ,,)	210
79 (4 ,,)	184
	Mean 197 (S.D. ±17·2)

irregularities, for in experiments under anaesthesia in 4 rabbits, section of both vagi had no effect upon the production of arrhythmia, upon the dose of noradrenaline required, or upon the level of blood pressure at which it appeared (Table II).

The Use of Different Pressor Agents

In order to determine whether noradrenaline produced these cardiac irregularities by virtue of some specific effect upon the myocardium or indirectly as a result of elevating the blood pressure, a further series of experiments was performed. When the dose of noradrenaline was reduced and the blood pressure maintained by adding posterior pituitary extract to the infusion fluid, the cardiac arrhythmia appeared at the same pressure (Table IIIA, Fig. 3). The importance of the level of pressure in the production of the ventricular arrhythmia could not be investigated further with posterior pituitary extract, since larger doses of posterior pituitary extract (100–200 mu./min.) neither produced the arrhythmia nor elevated the blood pressure to adequate levels. Injections of renin, however, raised the blood pressure to the necessary level and also provoked the cardiac arrhythmia (Table IIIA, Rabbit 91). The experiments with variations in pressor agents were performed initially in the anaesthetized animals and were extended and confirmed in the conscious animals (Table IIIB).

The evidence strongly suggested that the level of pressure was the essential feature in the production of ventricular arrhythmia, the pressor agent, and particularly noradrenaline, having no detectable action of its own. Further evidence to support this was obtained in 4 experiments when amyl nitrite, administered by inhalation, abolished the arrhythmia as it lowered the blood pressure. This is illustrated in Fig. 5, in which it may also be seen that the depression of the S–T segment of the electrocardiogram was not affected by the amyl nitrite.

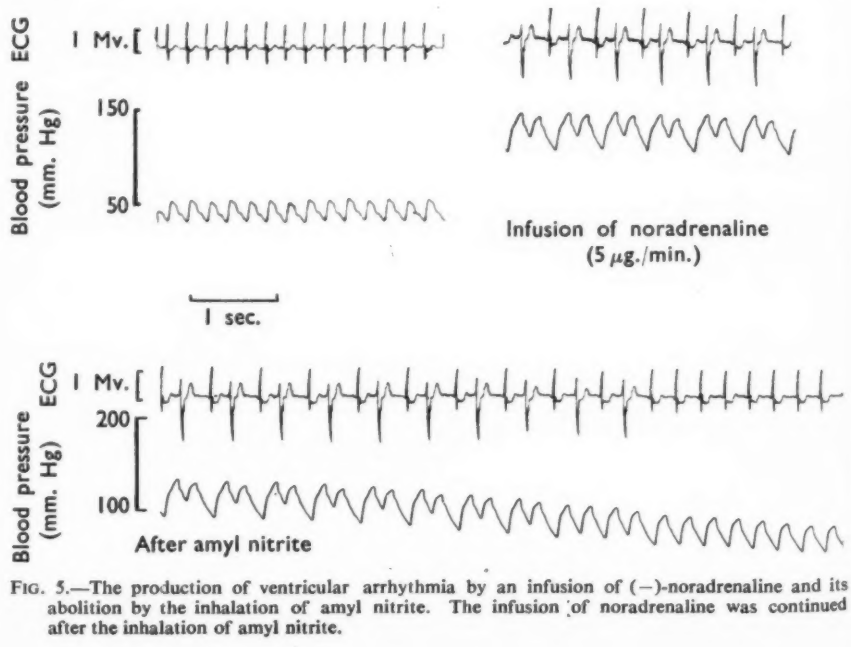

FIG. 5.—The production of ventricular arrhythmia by an infusion of (−)-noradrenaline and its abolition by the inhalation of amyl nitrite. The infusion of noradrenaline was continued after the inhalation of amyl nitrite.

TABLE II

THE EFFECT OF VAGAL SECTION UPON THE PRODUCTION OF VENTRICULAR ARRHYTHMIA IN ANAESTHETIZED NORMOTENSIVE RABBITS

Rabbit No.	Noradrenaline (μg./min.)	Average Pressure at which Arrhythmia Appeared (mm. Hg)	
		Vagi Intact	Vagi Cut
011	10	146	134
012	10	118	122
013	10	148	138
014	5	132	120

The Effect of Hypertension

Since the evidence pointed to the sudden elevation of pressure as the agent provoking the ventricular arrhythmia, a further series of experiments was performed in animals with experimental renal hypertension. In these comparative experiments precisely the same procedures were adopted in the normal and in the hypertensive animals. Conscious animals were used throughout. Noradrenaline was administered in progressively increasing doses by infusion into the marginal vein of the ear (Fig. 1) until the arrhythmia appeared.

The diastolic pressure at rest in the conscious rabbit ranged from 50–68 mm. Hg, and the mean pressure at which the arrhythmia appeared was 115 mm. Hg (S.D.±6) (Table I). In the hypertensive animals the diastolic pressure ranged from 84–102 mm. Hg, and the average pressure required to provoke the arrhythmia was 197 mm. Hg (S.D.±17.2); the difference between these means was statistically significant ($t=9$, $P<.001$).

DISCUSSION

Premature ventricular contraction and bigeminal rhythm was shown to follow a rise in systemic arterial pressure due to aortic compression by Rothberger and Winterberg (1910). This was confirmed by Levy (1914), who thought that the arrhythmia thus produced was different from the one evoked by adrenaline in cats sensitized with chloroform. The production of arrhythmias by adrenaline in sensitized dogs depends to some extent upon the level of blood pressure, since drugs which prevent the pressor response to adrenaline (F933) also suppress the arrhythmia (Shen, 1938). Evidence, however, accumulated that adrenaline-blocking drugs had a specific effect protecting against ventricular arrhythmias, and the part played by the level of blood pressure became obscure. It was shown in cats and rabbits that F933 not only suppressed ectopic ventricular beats produced by adrenaline but also those which were obtained with other agents or by electrical stimulation (Van Dongen, 1939). Furthermore, it could be shown that mechanical prevention of blood-pressure elevation could not completely prevent the arrhythmia produced by adrenaline after cyclopropane, whereas dibenamine could do so (Moe, Malton, Rennick, and Freyburger, 1948). This specific activity of dibenamine was confirmed by Nickerson and Nomaguchi (1949), who showed

TABLE III

THE EFFECT OF DIFFERENT DRUGS UPON THE LEVEL OF PRESSURE AT WHICH VENTRICULAR ARRHYTHMIA APPEARED IN NORMOTENSIVE RABBITS

Rabbit No.	Drugs	Average Pressure at which Arrhythmia Appeared (mm. Hg)
A. *Under anaesthesia*		
96 (2 occasions)	Noradr. 5 μg./min.	160
	Postpit. 50 mu./min.+ noradr. 2·5 μg./min.	144
06	Noradr. 10 μg./min.	162
93	Noradr. 5 μg./min.	128
	Postpit. 50 mu./min.+ noradr. 2·5 μg./min.	134
05	Noradr. 5 μg./min.	134 Abolished by amyl nitrite at 60
	Postpit. 50 mu./min.+ noradr. 2·5 μg./min.	130
91	Noradr. 5 μg./min.	140 Abolished by amyl nitrite at 100
	Renin 2 units	116 Abolished by amyl nitrite at 90
		Mean 136
B. *In the conscious state*		
92 (2 occasions)	Postpit. 50 mu./min.	130
	Renin 0·5 units	96
	Renin* + noradr. 2·5 μg./min.	104
83	Renin 0·5 units	120
	Renin* + noradr. 1 μg./min.	114 Abolished by amyl nitrite at 94
01	Renin 0·5 units	114
08	Noradr. 10 μg./min.	144
	Renin 0·5 units + noradr. 1 μg./min.	126
97	Renin 0·75 units	94
	Renin* + noradr. 1 μg./min.	110
		Mean 116

* The pressor effect of an injection of renin was very prolonged. It was therefore possible, during the gradual decline in blood pressure, to give an infusion of a small dose of noradrenaline.

that larger doses of dibenamine were necessary to prevent arrhythmias than were necessary to reverse the pressor response to adrenaline. It appeared therefore that elevation of the blood pressure was not essential for the production of arrhythmias by adrenaline after chloroform or cyclopropane, yet it remained a contributory factor, since doses of dibenamine just sufficient to prevent adrenaline arrhythmia became ineffective after mechanical elevation of the blood pressure.

The role of the level of pressure in the genesis of ventricular abnormalities was described by Dawes (1952) as one of 3 main variables capable of producing arrhythmias, but not as a crucial factor under all circumstances. The present studies have shown, in the rabbit at least, that the level of pressure may readily be the sole agent responsible for the production of ventricular arrhythmias. As far as can be seen from electrocardiographic evidence, the arrhythmias are the same as, though perhaps milder than, those occurring after chloroform or cyclopropane. In these experiments a direct action of noradrenaline could not be demonstrated. Since the arrhythmias were produced in animals under the influence of hexamethonium, and have also been reported to occur in the heart-lung preparation (Moe *et al.*, 1948), the level of blood pressure must be assumed to act directly upon the excitability of heart muscle, either through the excessive tension developed in systole, or by influencing diastolic size by increasing the systolic remainder. The protective effect of continued hypertension must be due to the muscular hypertrophy it initiates.

Recognition of the part played by the level of pressure in the production of ventricular irregularities might, if it can be shown to apply to man, be of practical value in the prevention or treatment of ventricular irregularities. This aspect deserves study, since it has been suggested that circulatory obstruction is a factor in the precipitation of ventricular fibrillation in cardiac surgery (Milstein and Brock, 1954). It is also possible that sudden elevation of pressure and ventricular fibrillation could be responsible for the sudden deaths occurring tragically at minor operations.

Summary

1. The circumstances responsible for the production of ventricular arrhythmia have been studied in the rabbit in the conscious state and under anaesthesia.

2. (−)-Noradrenaline, given by infusion to conscious normal rabbits in adequate doses, provoked the arrhythmia at an average blood pressure of 115 mm. Hg. The electrocardiogram showed that the arrhythmia was ventricular in origin, usually arising from a single focus and alternating with normal beats.

3. By using other pressor agents—posterior pituitary extract or renin—it was shown that the emergence of the arrhythmia depended upon the level of blood pressure and not upon the dose of noradrenaline.

4. The inhalation of amyl nitrite temporarily abolished the arrhythmia.

5. After the production of chronic hypertension the average level of blood pressure required to provoke the arrhythmia was 194 mm. Hg.

6. The possible practical implications of these observations are discussed.

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Brit. J. Pharmacol. (1956), **11**, 137.

A COMPARATIVE STUDY OF SUBSTANCE P FROM INTESTINE AND BRAIN

BY

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(RECEIVED SEPTEMBER 24, 1955)

In 1931 Euler and Gaddum extracted a smooth muscle stimulating factor—substance P—from intestinal muscle and brain tissue. As the active principles from these two sources had the same biological effect on different smooth muscle preparations, and the same thermostability and solubility in different solvents, they have been regarded as identical (Bjurstedt, Euler, and Gernandt, 1940; Pernow, 1953). This assumption, however, has never been subjected to closer investigation. Pernow (1953) purified the substance from intestine (PI) and that from brain (PB) separately by adsorption on aluminium oxide and partition chromatography on cellulose. In all steps of the purification the two principles behaved similarly, and the most purified preparations gave the same R_f-value on paper chromatograms using *n*-butanol/acetic acid/water (40:10:50) as solvent.

Since highly purified preparations of both PI and PB are now available, it seemed worth while to make a more detailed chemical and biological study of the two principles. The results of these experiments are presented in this paper.

METHODS

Extraction and Purification.—Substances PI and PB were obtained from cattle by boiling the minced tissue in water at *p*H 4 and precipitation after filtration with ammonium sulphate according to Euler (1942). Each substance was further purified by adsorption on aluminium oxide columns according to a method previously described (Pernow, 1953). The activity of the preparations was about 200 units/mg.

Counter-current Distribution was performed in a 25-tube steel Craig apparatus (Craig and Post, 1949), using *n*-butanol/acetic acid/water (40:10:50) as distribution phases, 8 ml. for each phase. The substances were studied separately. The dry weight of material was kept constant in all experiments, which were carried out in the following way: 1,000 units of PB in 1 ml. distilled water was inactivated with 0.05 mg. crystalline chymotrypsin in Tyrode solution (*p*H 8.3) at 38° C. overnight. After checking that all PB activity was destroyed, the enzyme was inactivated by boiling. The solution was then concentrated to dryness *in vacuo* and the residue dissolved in the lower phase of tube 0, which also contained 1,000 units of active PI. The distribution then started. In another experiment PI was inactivated in the same way and mixed with active PB. After a 25-tube run, 1 ml. of each lower phase was concentrated to dryness *in vacuo* at room temperature, dissolved in distilled water, and immediately tested on guinea-pig ileum.

Paper Electrophoresis was performed in the apparatus described by Kunkel and Tiselius (1951). Solutions of 0.05M-acetate buffer (*p*H 4.6–4.8), barbitone buffer (0.05M-barbitone sodium and 0.05M-HCl, *p*H 7.2–7.5), 0.05M-ammonium hydroxide-ammonium chloride buffer (*p*H 9.1–9.2), and glycine buffer (0.05M-NaOH, and 0.05M-glycine containing 2.68 g. NaCl/l., *p*H 10.1–11.7) were used. The voltage applied was 220 V D.C. After 2–5 hr. the paper (Whatman 20) was dried at room temperature. The localization of the active substances was done by cutting the paper into strips (2 × 0.5 cm.) and immersing each piece in an intestinal bath. In this way the activity was easily localized, since the substance, when present, was rapidly eluted from the paper and produced a slow contraction of the gut.

Paper Chromatography.—The paper used for the chromatograms was Grycksbo OB. The following solvents were used: *n*-butanol/acetic acid/water (40:10:50), *n*-butanol/ethanol/water (40:10:10), phenol/0.1N-HCl, pyridine/water (65:35). Substances PB and PI were chromatographed both separately and mixed during 15 hr. in a descending run at 23–25° C. The position of the substances on the filter paper was localized by the same method as described for the electrophoretic papers. This was successful only if the papers were run in an atmosphere of nitrogen, since the substances were inactivated during chromatography in air.

Enzymic Inactivation.—A crystalline chymotrypsin preparation, purchased at the Delta Chemical Works, Inc., N.Y., was used. To 40 units of PI and PB (0.20 mg.) separately in 1 ml. water was added 5 μg. chymotrypsin in 1 ml. solution, buffered to *p*H 8. The

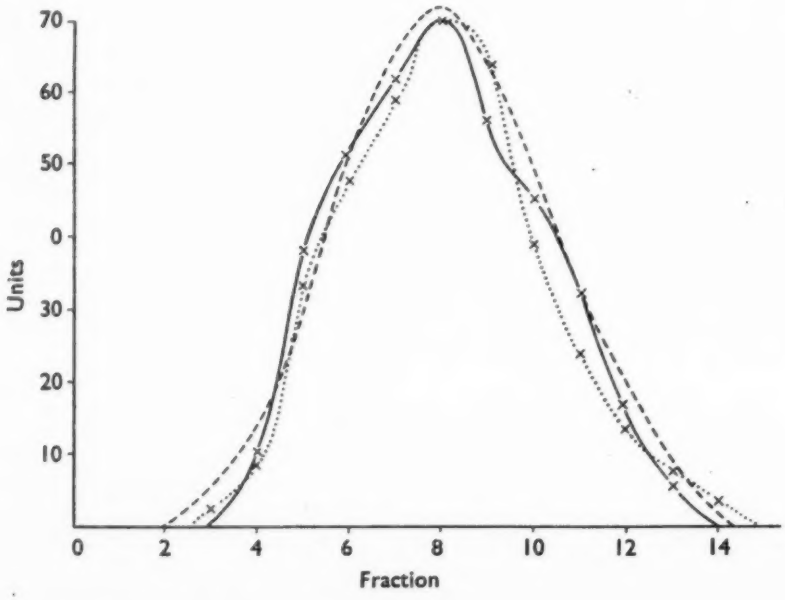

FIG. 1.—Counter-current distribution of substance P from intestine and brain. Solvent butanol/acetic acid/water (40:10:50). X——X, Substance P from brain; X···X, substance P from intestine; ----- K=0.5 (theoretical).

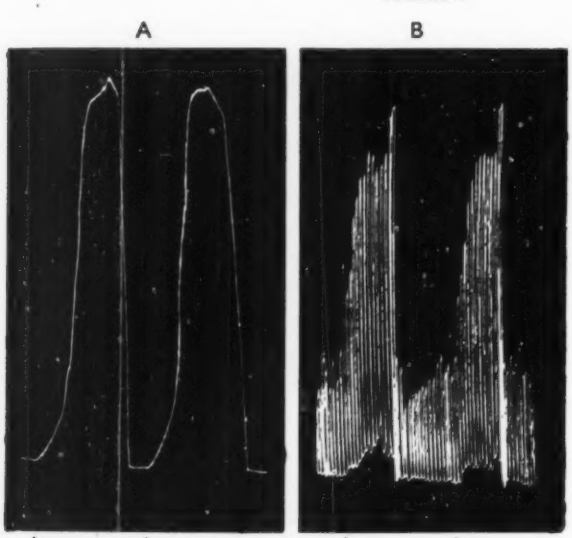

FIG. 2

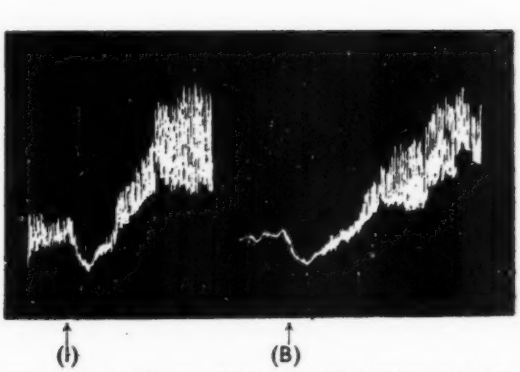

FIG. 3.—Rat duodenum. Effect of 2 units of substance P from intestine (I) and brain (B).

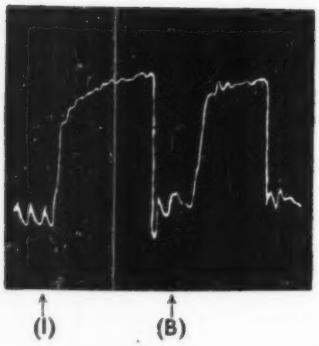

FIG 4

FIG. 2.—A, Isolated guinea-pig ileum. Bath volume 8 ml. Effect of 1 unit of substance P from intestine (I) and brain (B). B, Isolated rabbit jejunum. Bath volume 15 ml. Effect of 2 units of substance P from intestine (I) and brain (B).

FIG. 4.—Hen rectal caecum. Bath volume 15 ml. Effect of 1 unit of substance P from intestine (I) and brain (B).

FIG. 5.—Cat blood pressure. Effect of 40 units of substance P from intestine (I) and brain (B).

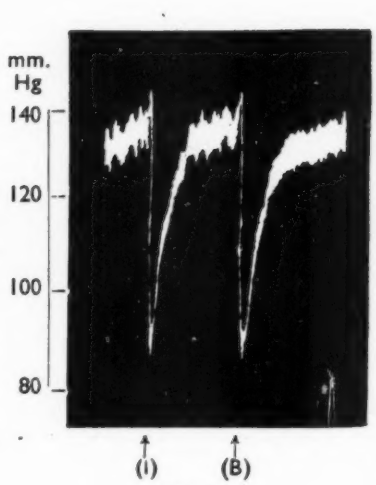

FIG. 5

incubation was performed at 38° C. It was always shown, in control experiments, that the gut was insensitive towards the pure enzyme.

Biological assay of substances PI and PB was on the isolated guinea-pig ileum, rabbit jejunum, rat duodenum, and hen rectal caecum, suspended in Tyrode solution. The guinea-pig and rat intestines were suspended in a 3 ml. bath, aerated with pure oxygen, and the rabbit and hen intestines in a 15 ml. bath aerated with 6.5% CO_2 in oxygen.

The vasodepressor actions of substances PI and PB were recorded from the carotid artery of rabbits anaesthetized with urethane (8 ml. 20% soln./kg., i.v.) and of cats anaesthetized with pentobarbitone sodium (35 mg./kg., i.p.).

Results

Counter-current Distribution.—Six experiments were performed, 3 with PB and 3 with PI in active form. In all distributions the greatest yield was obtained in tube 8, giving a distribution coefficient of 0.50 (Fig. 1). The yield of activity, about 40%, was the same for both substances.

Paper Electrophoresis.—Substances PI and PB were studied both separately and mixed. Within the *p*H-ranges used they showed the same mobility pattern. It was therefore not possible to separate them using this method.

Paper Chromatography.—Identical R_F values were obtained for substances PI and PB in the different solvents. For the purest preparations of both substances (2,000 u./mg.) these were 0.38 in *n*-butanol/acetic acid/water (40:10:50) and 0.92 in phenol saturated with 0.1N-HCl.

Enzymic Inactivation.—Earlier observations (Pernow, 1955) that substance P is rapidly inactivated with chymotrypsin were confirmed. Chymotrypsin (5 μg.) inactivated 40 units of PI and PB (0.25 mg.) in 30 min. No significant difference in the inactivation rate of PI and PB was found (Table I).

TABLE I

RATE OF INACTIVATION OF SUBSTANCE P FROM INTESTINE (PI) AND BRAIN (PB) BY CHYMOTRYPSIN

(40 units of substance P (0.20 mg.) incubated with 5 μg. of chymotrypsin at 38° C.)

Time (min.)	% Inactivation	
	PI	PB
1	0	0
3	25	30
8	40	40
12	60	65
16	70	75
21	85	
25		90
27	90	

Parallel Quantitative Biological Assays.—When substances PI and PB with the same degree of purity were tested on different isolated intestines the same height and type of contraction were always obtained. The ratio therefore was 1:1. The time course of the contraction (determined by measuring the time necessary for obtaining successive levels of contraction) was also the same for identical amounts of PI and PB (Figs. 2–4).

A fall in the blood pressure of about 20 mm. Hg was produced by 3–5 units in the rabbit and by 20–30 units in the cat. Identical effects on the blood-pressure recordings were obtained with equal amounts of PI and PB (Fig. 5).

Discussion

Before further investigation of the physiological actions of substances PI (from intestine) and PB (from brain) we thought it necessary to see if these substances were identical. Our comparative chemical and biological studies have given similar results for both, suggesting that the two substances are identical. Umrath (1953) found, however, that PI and PB were inactivated by extracts of nervous tissue at different rates: PB was destroyed by a proteolytic factor in nervous tissue 2–3 times faster than PI. Umrath's extracts of substance P, however, were prepared by boiling the tissue in a solution of 0.65% NaCl and 0.01% $CaCl_2$. It is known that such crude extracts also contain other smooth muscle stimulating substances such as 5-hydroxytryptamine and Darmstoff. The differences obtained by Umrath between PI and PB might therefore have been caused by the presence of such substances in varying proportions in the different extracts.

That the methods used in this paper are useful in separating substances with similar chemical and biological actions has recently been shown by Pernow and Rocha e Silva (1955) in a comparative study of bradykinin and substance P. These principles, both of which are polypeptides, inactivated by chymotrypsin, and both of which stimulate smooth muscle organs, could be completely separated by means of paper electrophoresis and counter-current distribution. A definite difference has also been demonstrated by comparing their actions on different isolated intestines.

Summary

1. Purified substance P preparations from cow's intestine and brain have been compared chemically and biologically.

2. It was not possible to distinguish chemically, physically, or biologically between substance P from intestine and that from brain.

3. It is therefore suggested that these substances are identical.

This investigation has been supported by a grant from the Therese and Johan Andersson Memorial Foundation. We wish to thank AB Astra, Södertälje, for the preliminary extraction of substances PI and PB.

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ANTICONVULSANT ACTION OF SOME ANTI-EPILEPTIC DRUGS IN MICE PRE-TREATED WITH *RAUWOLFIA* ALKALOIDS

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(RECEIVED OCTOBER 7, 1955)

It has been demonstrated that reserpine, in spite of its depressant action on the CNS, does not protect rats and mice against convulsions induced by electrical or chemical stimuli (Chen, Ensor, and Bohner, 1954; Tripod, Bein, and Meier, 1954). However, it protects normal rats against audiogenic seizures (Tripod *et al.*, 1954; Schneider and Earl, 1954), and protects chronically epileptic monkeys against convulsions provoked by prodding with a stick (Chusid, Kopeloff, and Kopeloff, 1955).

Chen, Ensor, and Bohner (1954), and Chen and Ensor (1954), further proved that reserpine, administered to mice, lowers the threshold of the CNS to electrical stimuli and suppresses the anti-electroshock effect of phenytoin and phenacemide, while enhancing their general depressant action.

The foregoing results suggest that reserpine has different points of action in the CNS: these are not yet clearly understood. Since the authors limited their investigations to reserpine and, generally, to electrically induced convulsions, we thought it worth while to extend the experiments by using some other convulsant drugs and other alkaloids of *Rauwolfia*. Moreover, since the foregoing authors generally used reserpine in doses that only provoke a slight sedative effect, we thought it opportune to employ *Rauwolfia* alkaloids and reserpine at the maximal doses permitted by their acute toxicities, in order to explore possible new properties not hitherto detected. The purpose of the researches has been to show whether *Rauwolfia* alkaloids, administered in high doses, (1) prevent or potentiate the convulsions elicited by three convulsant drugs having different types of action; (2) modify the spectrum of action of some anti-epileptic drugs having different types of action; (3) nullify, depress, or potentiate the anticonvulsant actions of these anti-epileptic drugs.

METHODS

The experiments were done on adult albino mice of both sexes.

The acute toxicities of the following drugs were determined: phenytoin sodium, troxidone, phenacemide, selected *Rauwolfia* alkaloids, called RA throughout this paper ("Rauserfia," Penick Brand, batch LMX 30–X 41659), and reserpine. The LD95, LD50, and LD5 (and the corresponding CD values) were calculated by the method of Litchfield and Wilcoxon (1949).

The CD95, CD50, and CD5 of the following drugs were determined: natural camphor, leptazol, and strychnine.

The anticonvulsant activities of the following drugs were determined: phenytoin sodium, troxidone, phenacemide, selected *Rauwo fia* alkaloids (RA) and reserpine. The ED95, ED50, and ED5 (anticonvulsant dose) were calculated for leptazol-induced seizures.

Camphor, leptazol, and strychnine were given intraperitoneally; phenytoin, troxidone, RA, and reserpine subcutaneously; and phenacemide by stomach tube.

Leptazol, strychnine, phenytoin, and troxidone were used in aqueous solution. Phenacemide was suspended in 10% w/v gum acacia solution. The solution of camphor was: natural camphor 0.5% w/v, acetone 2% v/v, polyoxyethylene sorbitan mono-oleate (Tween 80) 5% v/v. RA and reserpine were dissolved in: glacial acetic acid 2% v/v, Tween 80 6% v/v, N-NaOH 16%. A 1% or 0.5% solution of RA or reserpine was used in toxicity studies and a 0.1% solution in activity studies.

All activity studies were performed as follows: (1) Phenytoin, troxidone, phenacemide, RA, and reserpine, at 1/10 LD50, were administered 3 hr. before intraperitoneal injection of the convulsant drug at its CD95. Whenever the percentage of animals showing convulsions was sensibly below the expected 95%, the compound under examination was presumed to exert a protective action. With phenytoin, troxidone, and phenacemide, the same investigations were performed after administration of half the LD50. (2) Phenytoin, troxidone, phenacemide, RA, and reserpine at 1/10 LD50, were administered 3 hr. before intraperitoneal injection of the convulsant drug at its CD5. Whenever

the percentage of animals showing convulsions was sensibly above the expected 5%, the compound under examination was presumed to exert a facilitating action. With phenytoin, troxidone, and phenacemide, the same investigations were performed after administration of half the LD50. (3) Phenytoin, troxidone, and phenacemide at one half or one tenth their LD50, plus RA or reserpine at 1/10 LD50, were administered 3 hr. before the intraperitoneal injection of convulsant drugs, which were administered at the CD95 or CD5. The variations in the expected percentages of animals showing convulsions were evaluated as above.

Results

The acute toxicities of phenytoin, troxidone, phenacemide, RA, and reserpine are shown in Table I.

TABLE I

ACUTE TOXICITY IN MICE OF PHENYTOIN, TROXIDONE, PHENACEMIDE, RA, AND RESERPINE

(Phenacemide was given orally, the others subcutaneously. Confidence limits are for a probability (*P*) of 0·05)

Compound	LD50 (mg./kg.)	No. of Animals	Experimental Doses	
			1/2 LD50	1/10 LD50
Phenytoin sodium	160·0 (180·8–141·5)	60	80	16
Troxidone ..	>1,000	30	750	150
Phenacemide ..	About 3,000	50	1,000*	450*
RA	147·0 (155·8–138·7)	90	—	14·7
Reserpine ..	>200	25	—	30·0

* Phenacemide doses explained in text.

The mice treated with RA and reserpine were observed for only 24 hr., because in these animals, which showed a high degree of sedation, later deaths might not be directly correlated with the toxicity of the drugs. With other drugs the mice were observed for 48 hr. It was not possible to determine the exact LD50 for phenacemide, because its low acute toxicity and low solubility would have required the administration of very high doses. Moreover, no dose of reserpine higher than 200 mg./kg. and no dose of troxidone higher than 1,000 mg./kg. were given, since these doses were well tolerated by nearly all animals injected, and were considered sufficiently high to give a clear idea of the low acute toxicity of the drugs. The LD50's were arbitrarily presumed to be 50% greater than these highest doses administered. Throughout the experiments all the drugs, except phenacemide, were administered at 1/2 or 1/10 of the LD50, or of the presumed LD50. Phenacemide, on the contrary, was used in doses not directly correlated with its presumed LD50. The dose corresponding to 1/2 LD50 (1 g./kg.) was chosen as the highest dose causing mild sedation only, and the dose corresponding to 1/10 LD50 (450 mg./kg.) as the highest dose causing mild ataxia only. The doses used (reported in Table I) were never lethal.

The results obtained on testing phenytoin, troxidone, phenacemide, RA and reserpine as anticonvulsant drugs against camphor-, leptazol- or strychnine-induced seizures are reported in Tables II, III, and IV and are summarized below.

TABLE II

CONVULSANT ACTIVITY OF CAMPHOR (INTRAPERITONEALLY) IN MICE PRE-TREATED WITH ANTICONVULSANT DRUGS, RA, OR RESERPINE, AND WITH THEIR COMBINATIONS

(All pre-treatment drugs were given subcutaneously, except phenacemide, which was given orally 3 hr. before camphor)

Pre-treatment	% Mice (*) Convulsing at:	
	CD95	CD5
Control (0·5 ml. of vehicle for RA or reserpine) ..	90	0
Reserpine (1/10 LD50)	100	63
RA (1/10 LD50)	100	45
Phenytoin (1/10 LD50)	90	0
,, (1/2 LD50)	100	60
,, (1/10 LD50)+RA (1/10 LD50) ..	100	80
,, (1/10 LD50)+reserpine (1/10 LD50)	100	70
,, (1/2 LD50) +RA (1/10 LD50) ..	100	80
,, (1/2 LD50)+reserpine (1/10 LD50)	100	60
Troxidone (1/10 LD50)	90	0
,, (1/2 LD50)	100	0
,, (1/2 LD50)+RA (1/10 LD50) ..	50	0
,, (1/2 LD50)+reserpine (1/10 LD50)	80	0
Phenacemide (450 mg./kg.)	100	35
,, (1 g./kg.)	0	0
,, (450 mg./kg.)+RA (1/10 LD50) ..	30	0
,, (450 mg./kg.)+reserpine (1/10 LD50)	70	0
Phenacemide (1 g./kg.)+RA (1/10 LD50) ..	0	0
,, (1 g./kg.)+reserpine (1/10 LD50)..	0	0

(*) Each percentage is calculated on 10 or more animals.

TABLE III

CONVULSANT ACTIVITY OF LEPTAZOL (INTRAPERITONEALLY) IN MICE PRE-TREATED WITH ANTICONVULSANT DRUGS, RA, OR RESERPINE, AND WITH THEIR COMBINATIONS

(All pre-treatment drugs given subcutaneously, except phenacemide, which was given orally 3 hr. before leptazol)

Pre-treatment	% Mice (*) Convulsing at:	
	CD95	CD5
Control (0·5 ml. vehicle for RA or reserpine) ..	80	0
Reserpine (1/10 LD50)	100	100
RA (1/10 LD50)	100	84
Phenytoin (ED95)†	5	0
,, (ED95)+RA (1/10 LD50)	100	80
,, (ED95)+reserpine (1/10 LD50) ..	100	100
Troxidone (ED95)	5	0
,, (ED95)+RA (1/10 LD50)	100	0
,, (ED95)+reserpine (1/10 LD50) ..	100	30
Phenacemide (ED95)	5	0
,, (ED95)+RA (1/10 LD50)	20	10
,, (ED95)+reserpine (1/10 LD50) ..	20	50

(*) Each percentage is calculated on 10 or more animals.
† Anticonvulsant doses given in Table VI.

TABLE IV

LETHAL ACTIVITY OF STRYCHNINE (INTRAPERITONEALLY) IN MICE PRE-TREATED WITH ANTICONVULSANT DRUGS, RA, OR RESERPINE, AND WITH THEIR COMBINATIONS

(All pre-treatment drugs given subcutaneously, except phenacemide, which was given orally 3 hr. before strychnine)

Pre-treatment	% of Mice Killed* by Strychnine at:	
	LD95	LD5
Control (0·5 ml. vehicle for RA or reserpine) ..	100	0
Reserpine (1/10 LD50)	90	50
RA (1/10 LD50)	100	70
Phenytoin (1/10 LD50)	90	0
,, (1/2 LD50)	80	0
,, (1/10 LD50)+RA (1/10 LD50) ..	100	40
,, (1/10 LD50)+reserpine (1/10 LD50)	100	70
,, (1/2 LD50)+RA (1/10 LD50) ..	70	10
,, (1/2 LD50)+reserpine (1/10 LD50)	90	0
Troxidone (1/10 LD50)	100	0
,, (1/2 LD50)	80	0
,, (1/2 LD50)+RA (1/10 LD50) ..	100	0
,, (1/2 LD50)+reserpine (1/10 LD50)	80	0
Phenacemide (450 mg./kg.)	85	11
,, (1 g./kg.)	0	0
,, (450 mg./kg.)+RA (1/10 LD50) ..	90	0
,, (450 mg./kg.) + reserpine (1/10 LD50)	100	0
Phenacemide (1 g./kg.)+RA (1/10 LD50) ..	0	0
,, (1 g./kg.)+reserpine (1/10 LD50)	0	0

* Each percentage is calculated on 10 or more animals.

Camphor.—Camphor provoked only clonic convulsions. These were observed a few minutes after intraperitoneal injection and consisted, generally, in only one fit of very short duration. The mice, immediately after the injection, were placed on vertically suspended wire netting and, until the fit occurred, moved freely without falling. During the fit, preceded by a squeak, they were unable to cling to the net, and fell down. A linear relation was observed between the logarithms of the doses injected and the probits of animals which fell down (Fig. 1). The convulsant doses, CD95, CD50, and CD5, are given in Table V. The rapid occurrence of the fit, and the accuracy of the results obtained, depended on the vehicle used in preparing the camphor for injection. If an oily instead of an aqueous solution was used, the results were quite different. The vehicle used to dissolve the camphor had no effect on the behaviour of normal mice. The constancy of the CD95 and CD5 was frequently controlled. The values showed no significant differences in different batches of mice.

The convulsant activity of camphor was not modified by phenytoin at 1/10 LD50 and by troxidone at 1/2 or 1/10 LD50. The convulsant activity, however, was facilitated by phenytoin at 1/2 LD50 and, perhaps, by phenacemide at 450 mg./kg. Although this action of phenytoin might be due to the excitation which the compound elicited at the dose used, the action of phenacemide is not readily explicable since its effect at this dose was depressant. It appeared to possess a protective action at 1 g./kg.

Although normal mice generally had only one fit of very short duration after the administration of camphor, animals pre-treated with ineffective doses of anticonvulsant drugs had repeated fits.

RA and reserpine not only did not have any protective action, but facilitated the convulsions. The amount of solvent used had no effect alone. After RA or reserpine administration, convulsions were no longer only clonic: clonic-tonic convulsions similar to those induced by leptazol occurred, with maximal extensor spasm of the hind legs.

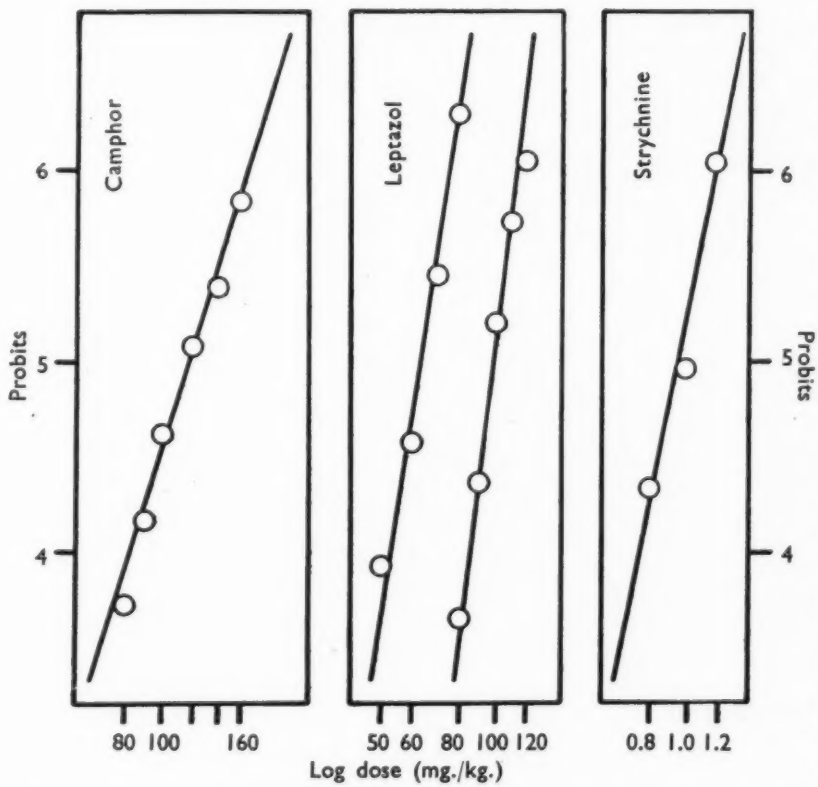

FIG. 1.—Showing the convulsant activity of camphor, leptazol and strychnine in mice. Abscissa, log dose in mg./kg. Ordinate, probits of animals convulsing.

TABLE V

CONVULSANT ACTIVITY IN MICE OF CAMPHOR, LEPTAZOL, AND STRYCHNINE ADMINISTERED INTRAPERITONEALLY

(Confidence limits $P=0.05$)

Compound	CD95 (mg./kg.)	CD50 (mg./kg.)	CD5 (mg./kg.)	No. and Batch of Mice Used
Camphor ..	213 (292–155)	118 (131–106)	66 (90·4–48·1)	100 B
Leptazol ..	89 (102–77·3)	66 (69·9–62·2)	49 (56·3–42·6)	110 A
" ..	130 (143–118)	102 (105–99)	81 (89·1–73·6)	85 B
Strychnine ..	1·45 (1·74–1·2)	0·98 (1·03–0·92)	0·66 (0·79–0·55)	75 B

Phenytoin at 1/10 LD50 as well as at 1/2 LD50 did not nullify the facilitating effect, on clonic convulsions, of the RA and reserpine. This effect, however, was completely nullified by troxidone and phenacemide. All three compounds completely nullified the tonic convulsions elicited by camphor in mice pre-treated with RA or reserpine. After the administration of phenytoin (1/2 LD50), troxidone and phenacemide, the mice showed ataxia and succeeded only with great difficulty in clinging to the net. Clinging to the net became impossible after treatment with RA or reserpine plus one of the three anti-epileptic drugs. The animals were therefore considered to be convulsing when they manifested clonic movements of the legs.

The depressant and sedative actions of RA and reserpine and of the three anti-epileptic drugs were mutually enhanced. The enhancement was very clear after phenacemide, and was small after troxidone. Only very slight enhancement was observed after phenytoin.

Leptazol.—The convulsions elicited by leptazol are of the clonic-tonic type. Only animals showing maximal, tonic extension of the hind legs were considered to be convulsing. The animals which died without showing maximal tonic extension were considered to be protected. There was a linear relation (Fig. 1) between the logarithms of the doses injected and the probits of animals showing maximal tonic extension. The convulsant doses (CD95, CD50, and CD5) are reported in Table V. During the investigations two different batches (batch A and batch B) of mice were used. The sensitivity of each batch to the convulsant drugs was evaluated separately. Their sensitivity to the convulsant activity of leptazol was different, corresponding to the two different probit/log dose lines in Fig. 1; whereas sensitivity to camphor and strychnine was not different. Sensitivity to the toxic action of phenytoin was unchanged.

The values of CD95 and CD5 for leptazol were frequently controlled. They did not vary within a single batch of mice.

Since phenytoin, troxidone, and phenacemide possess marked protective action against the tonic convulsions elicited by leptazol, it was thought opportune to calculate for each of the three compounds the doses which nullified completely the maximal extension of the hind legs provoked by the CD95 of leptazol. There was a linear relation (Fig. 2) between the logarithm of the dose employed and the probit of animals not showing maximal extension of the legs. The anticonvulsant doses, ED95, ED50, and ED5, are given in Table VI. The CD95 of leptazol was administered by the intraperitoneal route 3 hr. after the subcutaneous injection of the anticonvulsant drug. The difference in sensitivity to leptazol noticed between the two batches of mice found expression also in a difference in sensitivity to the anticonvulsant action of phenytoin. The values, ED95, ED50, and ED5, did not vary within a single batch of mice.

RA and reserpine did not protect against leptazol-induced seizures; they had, on the contrary, a marked facilitating action which was not counteracted by phenytoin but was partially counteracted by troxidone and phenacemide. The vehicle used for dissolving RA and reserpine for injection had no effect upon leptazol-induced convulsions.

In this group of experiments enhancement of the depressant and sedative effect of RA or reserpine and anti-epileptic drugs was again observed. Here, too, the greatest effect was obtained with phenacemide, the least with phenytoin.

Strychnine.—As the tonic convulsions provoked by strychnine are always followed by death, the lethal (LD95, LD50, and LD5) instead of the convulsant doses were calculated. The results are given in Fig. 1 and in Table V.

TABLE VI

ANTICONVULSANT ACTIVITY AGAINST LEPTAZOL-INDUCED SEIZURES OF SUBCUTANEOUS PHENYTOIN AND TROXIDONE AND OF ORAL PHENACEMIDE

(The anticonvulsant drugs were administered 3 hr. before the leptazol. The dose of leptazol was 130 mg./kg. i.p. except in the first experiment with phenytoin on mice of batch A, where it was 89 mg./kg.)

Compound	ED95 (mg./kg.)	ED50 (mg./kg.)	ED5 (mg./kg.)	No. and Batch of Mice Used
Phenytoin sodium	12·5 (19·0–0·82)	6·6 (7·5–5·7)	3·5 (5·3–2·3)	59 A
" "	6·6 (12·8–3·3)	1·9 (2·3–1·5)	0·54 (1·05–0·27)	80 B
Troxidone ..	490 (671–358)	270 (300–243)	150 (205–109)	85 B
Phenacemide ..	100 (171–58·4)	28 (34·4–22·7)	8 (13·7–4·6)	109 B

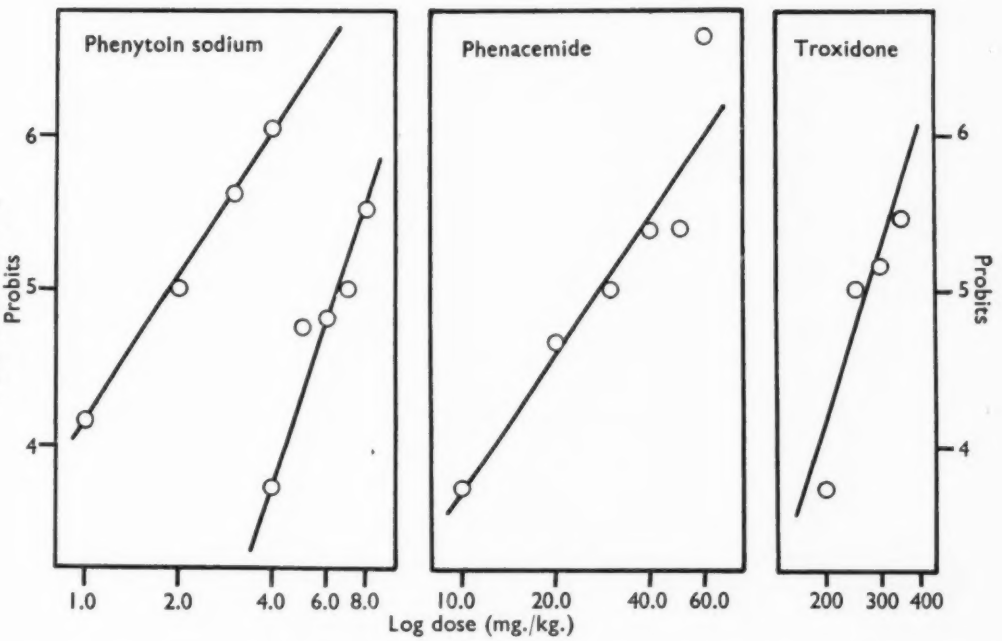

FIG. 2.—Showing the anticonvulsant activity of phenytoin, phenacemide and troxidone against leptazol - induced seizures. Abscissa, log dose in mg./kg. Ordinate, probits of animals not convulsing.

Among the anti-epileptic drugs tested, only phenacemide at a dose of 1 g./kg. exerted a protective action. RA and reserpine had a marked facilitating action, which could be inhibited by phenytoin (1/2 LD50), by troxidone, and by phenacemide. Enhancement in activity of the anti-epileptic drugs was never observed.

Deaths caused by strychnine, however, were delayed by anti-epileptic drugs as well as by RA and reserpine. While normal mice died generally from 30 to 60 min. after the injection of strychnine, pre-treated mice died many hours later. All the results reported were recorded approximately 20 hr. after the injection of strychnine.

DISCUSSION

The results obtained show that alkaloids of *Rauwolfia* do not protect against but, on the contrary, facilitate the convulsions elicited by camphor, leptazol, and strychnine.

It seems possible that they do this not only by lowering the threshold to convulsant drugs in the CNS (demonstrated by the fact that a dose almost inactive in normal mice became highly active in mice pre-treated with RA or reserpine), but also by facilitating the spread of stimuli from one region of the CNS to another—possibly from cortical to sub-cortical regions. Thus camphor provoked in normal mice only convulsions of the clonic type, whereas in pre-treated animals it also elicited tonic convulsions.

We supposed at first that a low blood sodium might account for the increased sensitivity of the CNS, for it is known that mice with a low blood-sodium level have an increased susceptibility to leptazol- or electrically-induced seizures (Swinyard, Brown, and Goodman, 1952; Swinyard, Schiffman and Goodman, 1955). This hypothesis, however, had to be discarded, because mice with a low blood sodium are no more susceptible to camphor-induced convulsions than are normal mice (the CD5 of camphor, given to 20 mice 2½ hr. after intraperitoneal administration of 1 ml./10 g. of a solution of 5.5% glucose, provoked convulsions, which did not have a tonic phase, in only two animals).

Since it is known that changes in the pituitary-adrenocortical axis can alter the excitability of the CNS, we then postulated that the adrenals were implicated in the facilitating action of *Rauwolfia* alkaloids. Previous studies demonstrated that adrenalectomy as well as hydrocortisone enhanced the susceptibility of mice and rats to convulsant drugs (Timiras, Woodbury, Despain, and Baker, 1955; Timiras, Woodbury, and Goodman, 1954), whereas DOCA decreased it (Timiras, Woodbury, and Goodman, 1954). ACTH has been shown to counteract the DOCA effect (Woodbury and Sayers, 1950). Furthermore, reserpine has been reported by Schneider (1954) to nullify the analgesic action of morphine in a similar way to ACTH and cortisone (Winter and Flataker, 1951), and has been shown to possess a stimulant action on the adrenals: in rats

the weight of the adrenals increases after reserpine treatment, whilst the thymus weight decreases (Gaunt, Renzi, Antonchak, Miller, and Gilman, 1954). Cronheim and Koster (1955) found that the adrenal ascorbic acid was depleted after reserpine treatment of rats. Mukherjee and Werner (1954) found a similar depletion in rats after treatment with " rauwolscine."

We accordingly injected 20 adrenalectomized mice (60 hr. after operation, and kept alive with NaCl 0.9%) with reserpine (30 mg./kg. s.c.). Six mice died within 3 hr. Thus adrenalectomized mice were more susceptible to the toxic effect of reserpine than were normal ones. To the 14 survivors, the CD5 of camphor was given intraperitoneally 3 hr. after the reserpine. Ten mice convulsed, and 6 of these died in tonic convulsions. On the whole, therefore, it can be said that adrenalectomized mice did not show any significant change in their susceptibility to the convulsant effect of camphor.

Summary

1. The anticonvulsant activity of soluble phenytoin, troxidone, phenacemide, reserpine, and a preparation containing mixed alkaloids of *Rauwolfia*, was evaluated in mice against camphor-, leptazol- and strychnine-induced convulsions. The anticonvulsant activity of combinations of the antiepileptic drugs with reserpine or mixed *Rauwolfia* alkaloids was also tested.

2. Reserpine and *Rauwolfia* alkaloids facilitate camphor-, leptazol-, and strychnine-induced seizures.

3. Reserpine and *Rauwolfia* alkaloids nullify the anticonvulsant activity of phenytoin, troxidone, and phenacemide against leptazol-induced convulsions, but do not nullify the activity of phenacemide against camphor- or strychnine-induced convulsions.

4. The facilitating action of reserpine and *Rauwolfia* alkaloids toward camphor- or leptazol-induced convulsions is partially counteracted by troxidone and by phenacemide. The facilitating action toward strychnine-induced convulsions is counteracted by troxidone, by phenacemide, and, partially, by phenytoin.

5. Camphor-induced convulsions in normal mice are exclusively clonic, but in mice pre-treated with reserpine or *Rauwolfia* alkaloids they become tonic. The anticonvulsant drugs studied suppress this action of *Rauwolfia*.

6. The facilitating action of *Rauwolfia* does not seem to be mediated by a lowering of the blood sodium level or by effects on the adrenals.

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PHARMACOLOGICAL STUDIES ON THE INFERIOR EYELID OF THE ANAESTHETIZED RAT

BY

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(RECEIVED OCTOBER 14, 1955)

In the present experiments it is shown that the contraction of the inferior eyelid of the anaesthetized rat provides a sensitive method for studying the effects of drugs acting on smooth muscle and sympathetic ganglia. This system has not hitherto been used in the rat, but has been studied in the cat and dog by Isola and Bacq (1946).

METHODS

Albino rats, approximately 200 g. in weight, were anaesthetized with pentobarbitone sodium (60 mg./kg.) intraperitoneally and fixed in the supine position to a heated metal platform which immobilized the head.

A horseshoe clamp, tightened from below and pressing against the upper canines, held the head down; lateral movement was prevented by an adjustable upright support on each side of the neck and pressing against the skull. The arms and legs of the animal were fixed by string to clamps.

A cannula was inserted into the trachea, and the cervical sympathetic trunk was dissected out under a dissecting microscope, tied peripherally, and cut. The nerve was kept immersed in warm paraffin. Stimulation was by Ag-AgCl electrodes through an RF isolation unit which allowed the animal to be completely dissociated electrically from the stimulating apparatus. It was essential to use an isolation unit; otherwise the effects of cervical sympathetic stimulation were often erratic and uncontrollable. Square wave stimulation was employed throughout at a pulse width of 250 μsec.

To record the eyelid movements a surgical silk thread was tied through the lower eyelid and fixed by plasticine to a mirror, suspended by a fine, taut wire. A slit of light was focused on the mirror and the leading edge of the reflected light beam allowed to fall on a photocell.

When the eyelid retracted, the beam of light was swept over a greater surface of the photocell so that a proportional movement of the lid was changed to a proportional amount of light illuminating the photocell. The output of the photocell was fed into a D.C. amplifier (Evershed & Vignoles, Ltd., London) matched to an ink writing recorder type PA 10M/A made by the same company. The amplification factor of the amplifier was kept constant throughout the experiments.

The cervical sympathetic trunk was stimulated with supramaximal voltage for periods of 5 sec. All drugs were given intravenously. For this purpose a small glass cannula, connected by a short length of rubber tubing to a mounted 5 ml. syringe, was inserted into the femoral vein. The drugs were injected through the rubber tubing by a fine needle (30 gauge) in a volume of 0.1–0.2 ml. and flushed into the vein with 0.2 saline from the syringe.

RESULTS

Stimulation of the cervical sympathetic for 5 sec. caused an immediate contraction of the inferior eyelid, the degree of contraction depending on the frequency of stimulation. In the experiment of Fig. 1, maximal contraction was observed with a frequency of 32 c/s. In most other experiments, maximal contraction was obtained with 25 c/s.

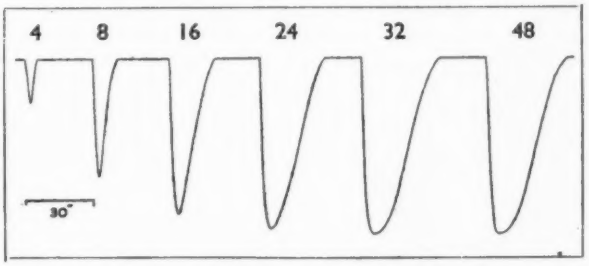

FIG. 1.—Rat, 205 g. Contractions of the inferior eyelid to varying frequencies of stimulation of the cervical sympathetic. Supramaximal voltage; pulse width 250 μsec., duration of stimulus 5 sec.

Adrenaline and noradrenaline produced a contraction of the inferior eyelid. Weight for weight adrenaline was about twice as active as noradrenaline. This is shown in the experiment of Fig. 2. For most animals the threshold dose for adrenaline was about 0.1 μg. (0.5 μg./kg.) and for noradrenaline 0.25 μg. (1.25 μg./kg.) computed as the base. When the doses were increased, height and duration of contraction increased concomitantly.

* During the tenure of a Fellowship in the Medical Sciences administered by the National Research Council for the Rockefeller Foundation.

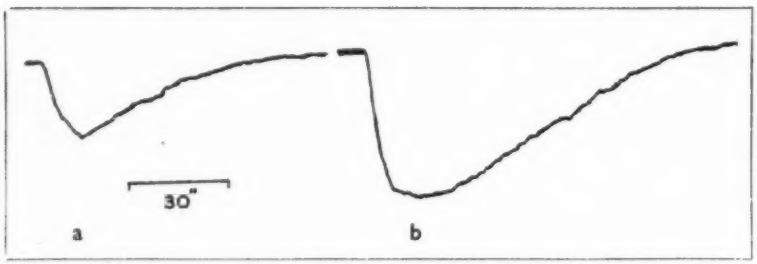

FIG. 2.—Rat, 195 g. Contractions of the inferior eyelid to intravenous administration of 0·5 μg. noradrenaline at a and to 0·5 μg. adrenaline at b computed as the base.

Acetylcholine, in doses of 10 μg. (50 μg./kg.) or more, caused a contraction of the inferior eyelid. This is shown in Fig. 3 which, in addition, illustrates the difference in response to a sequence of injections of the same dose of ACh. The contraction to a second injection of ACh was always greatly reduced, yet with subsequent administrations of the drug no further reduction in the response was usually seen.

Cocaine, in doses of 2–3 mg./kg., had no action of its own on the inferior eyelid, but enhanced both the responses to cervical sympathetic stimulation and to injections of adrenaline and noradrenaline. The great potentiation of the response to cervical sympathetic stimulation by cocaine is illustrated in Fig. 4. After cocaine, the responses to adrenaline and to noradrenaline became greater and persisted

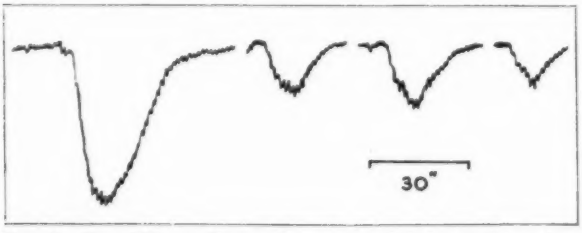

FIG. 3.—Rat, 205 g. Contractions of the inferior eyelid to four intravenous injections of 20 μg. acetylcholine chloride. One min. interval between each tracing.

longer, and a previously subthreshold dose became effective, so that it was often possible to obtain contractions of the inferior eyelid with 0.02 μg. (0.1 μg./kg.) adrenaline and 0.05 μg. (0.25 μg./kg.) noradrenaline. The potentiating effect of cocaine lasted for about 1 hr. It was not possible to detect whether there was a difference between the potentiation of adrenaline and noradrenaline by cocaine.

Phentolamine (Rogitine), when injected in doses of 1 mg. (5 mg./kg.), rendered the lower eyelid insensitive to stimulation of the cervical sympathetic and to adrenaline and noradrenaline. The effect was immediate and lasted for about ½ hr.

Tetraethylammonium chloride (TEA), *hexamethonium chloride* (C6), and *Ro* 2–2581 [(+)-3,4(1′,3′-dibenzyl-2′-keto-imidazolido)-1,2-trimethylene thiophanium chloride], a short-acting thiophanium ganglion-blocking agent, abolished the effect of sympathetic stimulation on the eyelid. Higher doses of all three, however, were required to produce this effect than in corresponding experiments on the nictitating membrane of the cat. A typical result with Ro 2–2581 is shown in Fig. 5; the injection of 2 mg./kg. greatly reduced, but did not abolish, the effect of cervical sympathetic stimulation.

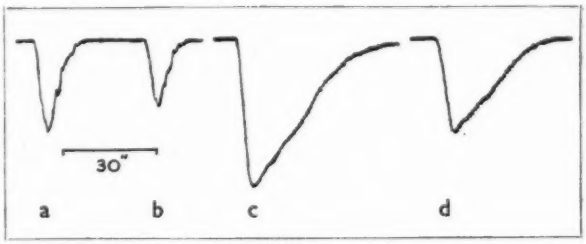

FIG. 4.—Rat, 200 g. Contractions of the rat's inferior eyelid to 5 sec. periods of stimulation of the cervical sympathetic. Potentiation of the response by cocaine. Stimulation at 4 c/s at a and c, and at 2 c/s at b and d; 250 μsec. pulse width. Five min. before c, intravenous injection of 0·4 mg. cocaine hydrochloride.

The action was over in 3 min. In the cat, 2 mg./kg. would have produced complete block lasting for over 15 min. (Randall, Peterson and Lehmann, 1949). In a 200 g. rat complete block occurred only after 5 mg./kg. and never lasted longer than 5 min. TEA produced complete block in doses of the order of 25 mg./kg. and hexamethonium in doses of 5 mg./kg. The block produced by TEA was over within a few minutes; that produced by hexamethonium lasted for about 15 min.

Eserine (0.5 mg./kg.) had no action on the lower eyelid and did not affect the response to sympathetic

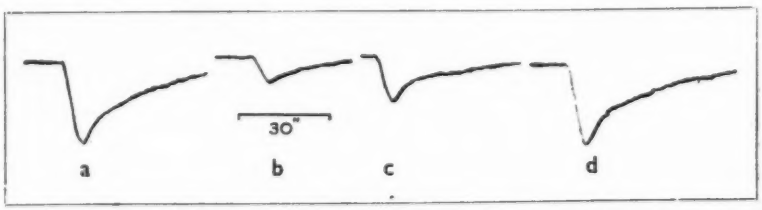

FIG. 5.—Rat, 210 g. Contractions of the inferior eyelid to 5 sec. periods of stimulation (at 4 c/s, 250 μsec.) of the cervical sympathetic before (a) and 1 min. (b), 2 min. (c) and 3 min. (d) after an intravenous injection of 0·4 mg. of Ro 2–2581.

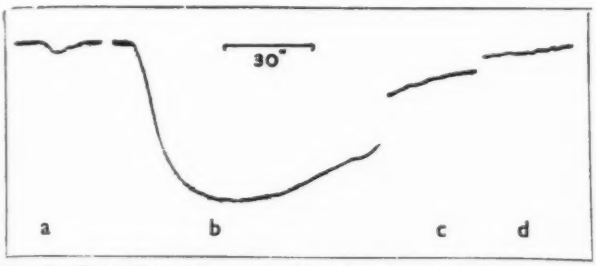

FIG. 6.—Rat, 200 g. Contractions of the inferior eyelid to intravenous injections of 10 μg. acetylcholine chloride, before (a) and after (b) an intravenous injection of 0·1 mg. eserine sulphate. Between (b) and (c), and (c) and (d), one minute intervals.

stimulation, but greatly enhanced the response to ACh (Fig. 6).

Atropine, in small doses (0.5 mg./kg.), rendered the eyelid muscle insensitive to ACh, but did not affect its response to sympathetic stimulation, adrenaline, or noradrenaline. Larger doses of atropine (2 mg./kg.) caused relaxation of the eyelid. This effect was small, but occurred regularly. It is illustrated in Fig. 7 by the fact that the record at C starts at a slightly higher point in the tracing than at A and B. The figure further illustrates that, in larger doses, atropine reduced, to a slight extent, the responses both to adrenaline and to cervical sympathetic stimulation. The response to noradrenaline was equally reduced under these conditions. Usually, the effect of atropine lasted for less than $\frac{3}{4}$ hr.

(+)-*Tubocurarine chloride* in doses which produce arrest of respiration had no action on the eyelid, nor did it affect the response to adrenaline or noradrenaline; but it did cause a slight diminution of the response to stimulation of the cervical sympathetic. This reduction is most likely due to the ganglion-blocking action of curare.

DISCUSSION

The results suggest that the sympathetic innervation of the smooth muscles of the eyelid of the rat consists solely of adrenergic fibres, and that these muscles receive no cholinergic, postganglionic, sympathetic fibres. This conclusion is based on the findings that cocaine potentiates and phentolamine blocks the effect of sympathetic stimulation to the eyelid; and that neither eserine, nor small doses of atropine which abolish the effect of injected acetylcholine, influence the response to sympathetic stimulation. The ability of atropine in large doses to depress the response cannot be taken as evidence for a cholinergic nerve supply, since it is known (Kuroda, 1924; Schilf, 1927; Feldberg, 1931; Gaddum, 1936) that large doses of atropine have an unspecific depressant effect on smooth muscles.

The finding that the rat eyelid responds to relatively small doses of adrenaline or noradrenaline, particularly after cocaine, and discriminates well between different doses, renders it a suitable preparation for the assay of small amounts of adrenaline or noradrenaline and probably of other sympathomimetic amines as well. The amounts of adrenaline which could be assayed with this method were of the order of 0.02–0.1 μg. The preparation should also prove useful when testing ganglion-blocking agents; because of the smallness of the animal—usually 200 g.—it would be possible to assay such agents when available only in small amounts.

SUMMARY

1. The contractions of the inferior eyelid of the anaesthetized rat have been recorded. Contractions were produced by stimulation of the cervical sympathetic and by intravenous injection of adren-

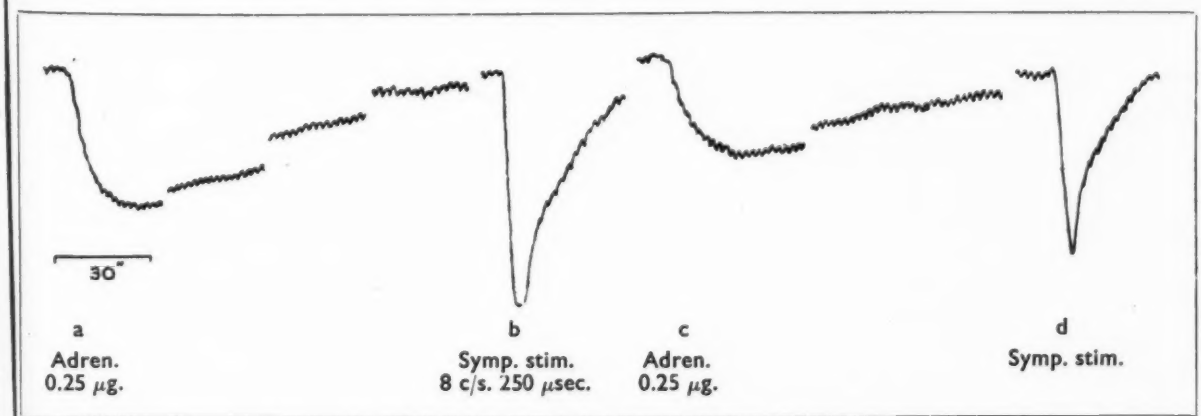

FIG. 7.—Rat, 195 g. Contractions of the inferior eyelid to intravenous injections of 0·25 μg. adrenaline, computed as base (at a and c); and to 5 sec. periods of stimulation of the cervical sympathetic (at b and d) at 8 c/s, 250 μsec. pulse width. Intravenous injection of 0·4 mg. atropine sulphate immediately after b and 5 min. before c.

aline, noradrenaline, and acetylcholine. The contractions to sympathetic stimulation were enhanced by cocaine and abolished by phentolamine, hexamethonium, tetraethylammonium and Ro 2–2581.

2. This preparation is useful for assaying sympathomimetic amines, their antagonists, and ganglion blocking agents—particularly when only a small supply of material is available.

I am indebted to Dr. W. Feldberg for his continued interest during this investigation, to Dr. Laurence Malcolm for help and advice in setting up the experimental arrangement, and to Dr. Elmer Sevringhaus, of Hoffman La Roche, for a generous supply of Ro 2–2581.

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Brit. J. Pharmacol. (1956), **11**, 151.

THE MECHANISM OF ACTION OF PARALDEHYDE AND METHYLPENTYNOL ON NEUROMUSCULAR TRANSMISSION IN THE FROG

BY

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(RECEIVED NOVEMBER 25, 1955)

Paraldehyde and methylpentynol block neuromuscular transmission (Quilliam, 1955), and the object of the present study was to analyse the mode of action of the two drugs using electrical methods. The experimental evidence obtained suggests that the neuromuscular blocking activity of paraldehyde and of methylpentynol can be accounted for by their action in decreasing ACh release. Some of the results were communicated to the British Pharmacological Society in July, 1954, and in January, 1955.

METHODS

The *extensor longus digiti IV* muscle of the frog was used in all experiments. The preparation was carefully dissected with its nerve and soaked in frog-Ringer solution containing 1×10^{-6} neostigmine for 30 min. before beginning each experiment. Only uninjured preparations were used, since even one injured fibre gives rise to large injury potentials. The muscle was mounted vertically in a bath with the proximal nerve-free end uppermost (Fatt, 1950). Recordings were made through silver-silver chloride electrodes which fed through balanced cathode followers and a D.C. amplifier (Copeland, 1952) to an oscilloscope equipped with a camera.

End-plate potentials were recorded in the manner described by Del Castillo and Stark (1952), square wave stimuli being applied to the nerve through platinum electrodes. A concentration of between 1.6×10^{-6} and 3.2×10^{-6} tubocurarine ensured the production of a pure end-plate potential (e.p.p.) by nerve stimulation.

The depolarizing action of a drug was studied by measuring the potential produced in the region of maximum end-plate density. To avoid errors in localization of this region, a moving fluid electrode was used to record the potential distribution along the whole muscle surface (see Fatt, 1950; Del Castillo and Stark, 1952). Increasing concentrations of a depolarizing drug, such as ACh, produce progressively larger depolarizations of the end-plate region, and a dose-response curve can be drawn of the end-plate depolarization against ACh concentrations (Fatt, 1950).

RESULTS

The Effect of Paraldehyde on the End-plate Potential

Paraldehyde greatly reduced the size of the e.p.p. in every experiment. The e.p.p. (produced by nerve stimulation in the presence of the blocking concentration of tubocurarine) remains constant for many minutes, and the action of various concentrations of paraldehyde can thus be accurately measured. The relationship between the magnitude of the e.p.p. and paraldehyde concentration is plotted in Fig. 1. The effect of paraldehyde was rapidly and usually completely reversed by washing. After treatment with tubocurarine as described, the e.p.p. was completely abolished by the addition of 2×10^{-3} paraldehyde, which was the minimum

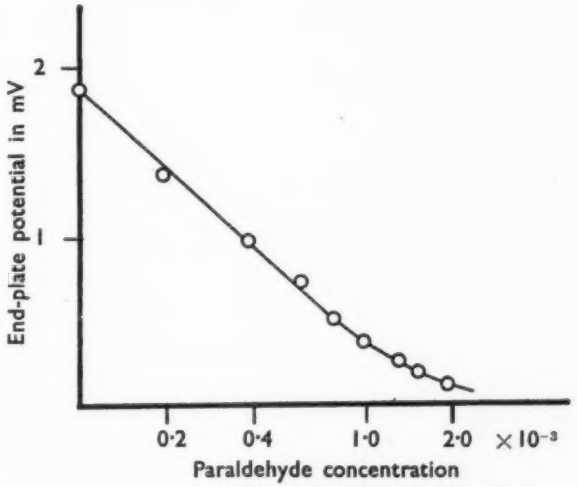

FIG. 1.—To show the relation between the magnitude of the tubocurarine end-plate potential in mV and the log of the paraldehyde concentration in the Ringer solution bathing the *M. extensor longus digiti IV* of the frog.

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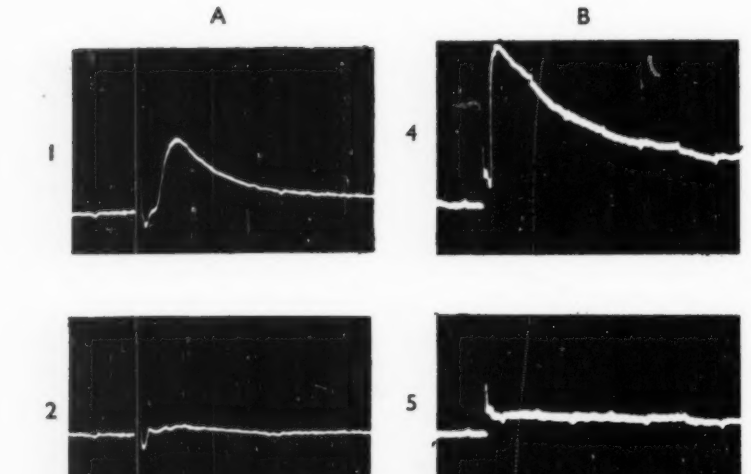

FIG. 2.—The abolition of the end-plate potential recorded from the *M. extensor longus digiti IV* of the frog by that concentration of paraldehyde which had to be attained before neuromuscular block was complete in a fresh muscle. A, Records made in the presence of 3.1×10^{-9} tubocurarine. 1, Before, and 2, during exposure to 2×10^{-3} paraldehyde; 3, recovery of e.p.p. after washing. B, Records made in the presence of 4×10^{-7} ACh. 4, Before, and 5, after soaking in 2.4×10^{-3} paraldehyde; 6, recovery of e.p.p. after washing. In A, note the small downward deflection preceding the e.p.p., due to the nerve action potential which was unaltered by paraldehyde.

concentration of paraldehyde required to block neuromuscular transmission when previously applied to the fresh preparation (Fig. 2A).

Paraldehyde had the same effect when a drug other than tubocurarine was used to demonstrate the e.p.p. Thus in two experiments ACh in concentrations between 2 and 4×10^{-7} was used to produce neuromuscular block (Del Castillo and Stark, 1952); the e.p.p. after nerve stimulation was reversibly abolished by the blocking concentration of paraldehyde (Fig. 2B).

The abolition of the e.p.p. by paraldehyde may be due to one or more of the following mechanisms: (1) block of transmission in the nerve, (2) prevention of the release of ACh from the motor nerve endings, (3) decrease in the sensitivity of the end-plate region to ACh, (4) persistent depolarization of the end-plate region, or (5) decrease in the excitability of the muscle fibres. It was not possible to study directly the release of ACh by the motor nerve endings in this preparation, but the effect of paraldehyde on the nerve, on the end-plate region, and on the muscle membrane could be measured.

Effect of Paraldehyde on Nerve

In all experiments, the nerve action potential was measured before and after adding the blocking concentration of paraldehyde. No change was observed in the amplitude or form of

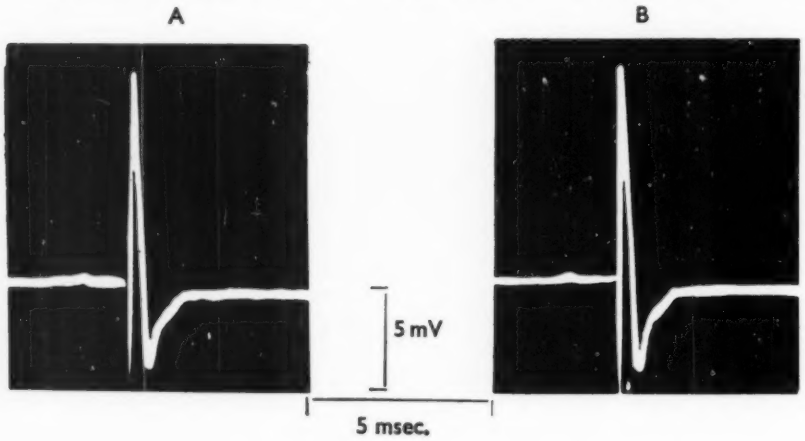

FIG. 3.—The action potential recorded from the motor nerve to the *M. extensor longus digiti IV* of the frog: A, before, and B, after soaking in that concentration of paraldehyde which caused a complete neuromuscular block in the preparation.

the potential (Fig. 3). In some experiments, it was possible to record the nerve action potential as a small downward deflection preceding the e.p.p. Such an experiment is illustrated in Fig. 2A, in which the nerve action potential was uninfluenced by paraldehyde.

Effect of Paraldehyde on the Depolarization Produced by ACh

Increasing concentrations of ACh in the fluid bathing a muscle produced progressively greater depolarizations of the end-plate region. When the e.p.p. reached a critical level, muscle action potentials were initiated. A typical dose-response curve is shown in Fig. 4 (solid circles), which relates the peak depolarization of the end-plate region to ACh concentration. Such a curve is an index of the sensitivity of the end-plate region to added ACh. The effect of paraldehyde on the sensitivity to added ACh has been studied, therefore, in the following way. The ACh dose-response curve was determined in a fresh preparation. Thereafter, the minimum concentration of paraldehyde which produced neuromuscular block—so that nerve stimulation then gave a pure e.p.p.—was determined. The muscle was then soaked in this concentration of paraldehyde and the ACh dose-response curve was measured again. The blocking concentration of paraldehyde had little or no effect upon the position or shape of the curve (open circles in Fig. 4). Furthermore, the threshold concentration of ACh which must be added for the initiation of *action potentials in the muscle* was unaltered by the

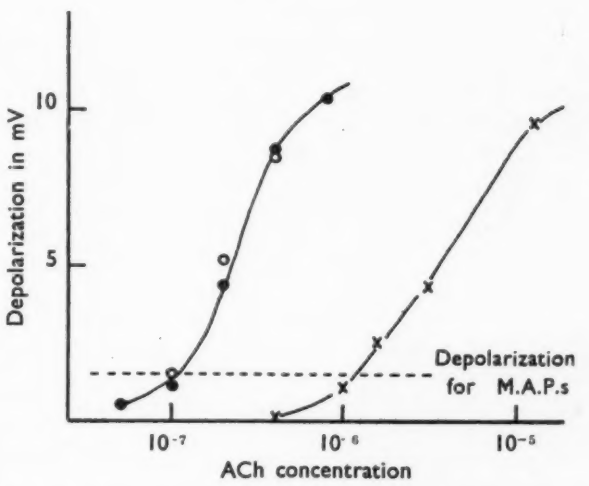

FIG. 4.—*M. extensor longus digiti IV.* Acetylcholine dose-depolarization response curves. Solid circles, normal muscle; open circles, after soaking in paraldehyde (2×10^{-3}); and crosses, after treating with tubocurarine (3.1×10^{-6}). The depolarization required to initiate muscle action potentials is indicated by the dotted line.

blocking concentration of paraldehyde (dotted line in Fig. 4). This is quite different from the action of a competitive blocking agent such as tubocurarine. In the same preparation, the blocking concentration of tubocurarine shifted the ACh dose-response curve to the right by about one $\log_{10}$ unit (crosses in Fig. 4). This shows that, to produce a given depolarization in the presence of tubocurarine, about ten times more ACh must be added to the fluid bathing the muscle. Consequently the pro-

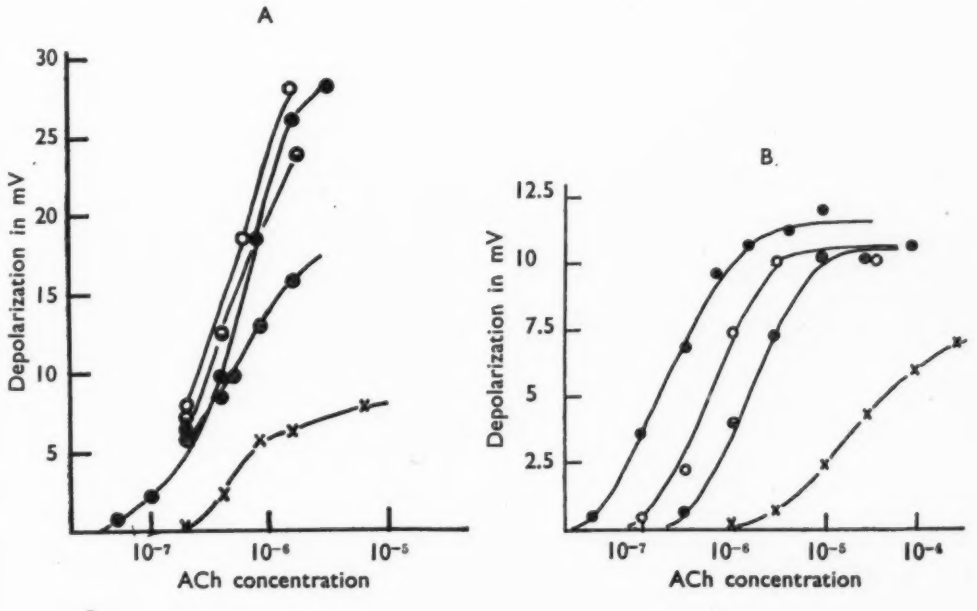

FIG. 5.—Dose-response curves as in Fig. 4. A, in paraldehyde (O, 5×10^{-4}; ◒, 1×10^{-3}; ⊙, 2×10^{-3}; ×, 3×10^{-3}; ●, control). B, in tubocurarine (O, 5×10^{-7}; ⊙, 10^{-6}; ×, 10^{-5}; ●, control).

duction of neuromuscular block and the abolition of the e.p.p. do not seem to be due to a post-junctional action of paraldehyde.

High concentrations of paraldehyde, on the other hand, did modify the dose-response relation but not in the same way as did tubocurarine. Increasing paraldehyde concentrations reduced the maximum depolarization attained, but produced only a slight shift to the right (Fig. 5A). Some depression of the maximum was seen with the blocking concentration (2×10^{-3}), but this effect was reversible. The gross depression of the maximum with higher paraldehyde concentrations (e.g. 3×10^{-3}) was not reversed even by prolonged washing. Even the highest concentrations of paraldehyde used did not depolarize the muscle membrane at rest.

Increasing concentrations of tubocurarine tested in the same way shifted the ACh dose-response curve progressively to the right (Fig. 5B). As high a concentration as 1×10^{-5} was needed before some depression of the maximum, of the type seen with paraldehyde, occurred. In none of our experiments with tubocurarine alone was any depolarization of the muscle membrane seen under resting conditions.

Methylpentynol

In concentrations which caused neuromuscular block, methylpentynol suppressed the e.p.p. seen after tubocurarine or ACh. After exposing a foot muscle to that concentration of methylpentynol which caused neuromuscular block (1.2×10^{-3}), the depolarizing action of small doses of ACh was unimpaired whereas larger doses were about half as active as in a normal preparation (Fig. 6). The degree of depolarization necessary to set up muscle action potentials in response to added ACh was not modified by methylpentynol.

While in most muscles methylpentynol in a concentration of 1×10^{-3} produced no depolarization, a few preparations did show evidence of a slight depolarization. However, unlike paraldehyde, higher concentrations of methylpentynol, for example 7×10^{-3}, gave a marked and persistent depolarization which was greatest in the region of maximum end-plate density and was not reversed by washing.

Procaine

When a muscle was soaked in a concentration of procaine which caused neuromuscular block, the ACh dose-response curve was shifted to the right but not so far as with a blocking concentration of tubocurarine (Fig. 7). The blocking concentration of procaine had no effect upon the size or shape of the nerve action potential. The competitive effect precluded critical study by our methods of any effect which procaine might have had upon ACh release at the neuromuscular junction.

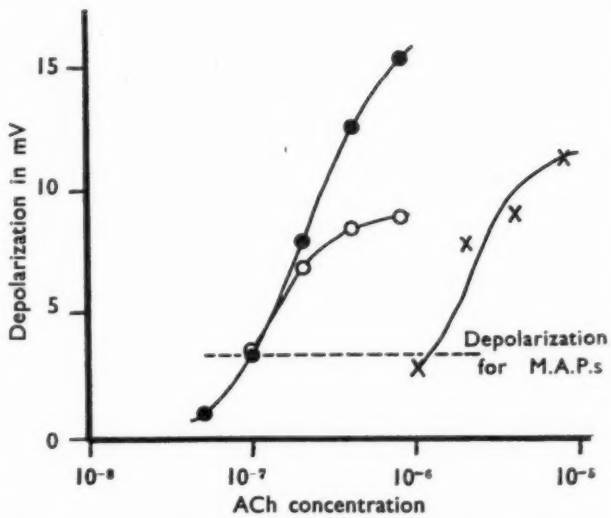

FIG. 6.—Dose-response curves as in Fig. 4. Solid circles, normal muscle; open circles, after soaking in methylpentynol (1.2×10^{-3}); and crosses, in tubocurarine (1.5×10^{-6}).

DISCUSSION

The results reported in this paper show that paraldehyde and methylpentynol, in concentrations

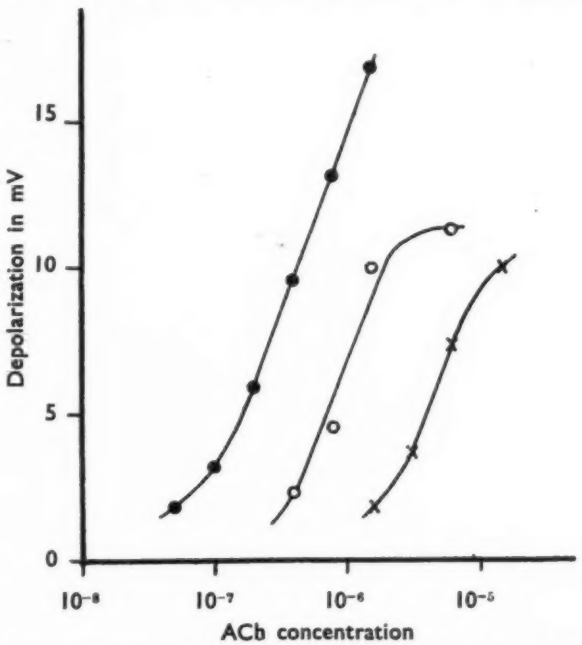

FIG. 7.—Dose-response curves as in Fig. 4. Solid circles, fresh muscle; open circles, after soaking in procaine (5.5×10^{-6}); and crosses, in tubocurarine (2.25×10^{-6}).

which block neuromuscular transmission, suppress the e.p.p. observed after tubocurarine or ACh. The actions of paraldehyde and methylpentynol differ from those of decamethonium and tubocurarine respectively in that they neither depolarize the muscle cell nor compete for ACh receptors. The two hypnotic drugs appear neither to modify the response of the muscle cell to added ACh nor to interfere with conduction of nerve impulses in nerve trunks. Thus, it seems that they must abolish the e.p.p. by reducing the amount of ACh released by nerve impulses at the terminals of the motor nerves, and that they owe their neuromuscular blocking activity to this action. Their effect does not damage the preparation and is readily reversible.

In the experiments with the blocking concentration of procaine, the dose-response curve was shifted to the right, but this shift was not as great as that produced by the blocking concentration of tubocurarine in the same muscle. In part at least, the neuromuscular blocking activity of procaine can be accounted for by a competitive action, and this effect precluded further analysis by our electrical methods of the mode of action of this drug.

Summary

1. Paraldehyde and methylpentynol reversibly block neuromuscular transmission in a frog nerve-muscle preparation.

2. Using electrical recording methods, it was found that both drugs abolished the end-plate potential set up by nerve stimulation in preparations in which transmission was blocked with tubocurarine or ACh.

3. The blocking concentrations of paraldehyde and methylpentynol do not, however, (*a*) block transmission in the nerve, (*b*) depolarize the end-plate region, (*c*) act like tubocurarine or (*d*) decrease the excitability of the muscle membrane.

4. It was concluded that the neuromuscular blocking activity of paraldehyde and methylpentynol could be accounted for only if they reduced the amount of ACh released by nerve impulses. This action does not damage the muscle cell and is reversible.

5. Procaine, in contrast, acted in a similar manner to tubocurarine by reducing the sensitivity of the end-plate region to ACh. It is suggested that the blocking action of procaine at the neuromuscular junction can be accounted for, in part at least, by its competitive action.

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THE ESTIMATION OF THE SAFE DOSE

BY

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(RECEIVED DECEMBER 1, 1955)

Small quantities of toxic substances may be present in food, either because they have been deliberately added to improve the appearance of the food or because they have been used as insecticides or for various other reasons. This can do no harm if the quantities are small enough, and it is sometimes necessary to decide what is the maximum quantity that can be regarded as quite safe. For some of the older poisons evidence on this point may be available from observations on men. The doses of arsenic used by numerous poisoners have been estimated, and from a consideration of the effects produced it is possible to determine the lowest recorded toxic dose; a dose somewhat lower than this can be regarded as safe. With new poisons this method is not immediately applicable, and it is sometimes necessary to get indirect evidence from experiments on animals. Such experiments are difficult to design (Barnes and Denz, 1954b). Preliminary work may show that a given dose has little effect, and experiments are then sometimes undertaken to show that a smaller dose has no effect at all. If 100 rats are fed on a suitable diet for 6 months without obvious ill effect, it may seem reasonable to suppose that the diet is not very toxic, but it is clear that a diet which killed 1% of rats might easily fail to kill any of the particular rats chosen for the test. The probability that any one rat will survive is 0.99 and the probability that 100 rats will all survive is $0{\cdot}99^{100}$. Therefore the probability that this test would fail to detect this toxicity would be 0.99^{100}, or about 0.37, but a diet which killed 1% of the people who ate it would be a very undesirable diet. Some allowance can be made for this weakness in the argument by setting the limit at 1/10 or 1/100 of the quantity shown to be safe, but such factors of safety are a very arbitrary form of extrapolation, and if the variation of the response to the drug is high, they may not be as safe as they seem to be.

Another difficulty about experiments of this type lies in the fact that occasional deaths are liable to occur in a colony of rats even when no poisons are given. The maintenance of 100 control rats without poisons adds considerably to the labour of the experiments and to the error of the result if deaths occur in both groups. Such experiments are costly and dull and the evidence they provide cannot be satisfactory. The present paper discusses alternative types of experiment in which safe doses are determined by extrapolation from the results of experiments with doses which have measurable effects. Extrapolation is not a reliable procedure, but in the present case it is inevitable, and it is thought that the arguments given below are at any rate preferable to the vague form of extrapolation outlined above.

A suitable method of calculation was described by Gray, Trevan, Bainbridge, and Attwood (1931), who measured the relation between the dose of stibamine glucoside and the mortality of mice, and suggested that the maximum tolerated dose should be defined in terms of an arbitrary small mortality, and that it should be calculated by extrapolation on the assumption that the logarithm of the individual lethal dose was normally distributed. Calculations of this type are easily made to-day, and it is known that the basic assumption is generally true (Gaddum, 1933, 1953). Gray *et al.* suggested that the dose which would kill 1 mouse in 742 should be called the maximum tolerated dose. This corresponds to 3 standard deviations from the mean. It is now proposed that the safe dose be calculated from the point corresponding to 6 deviations from the mean. This would be expected to kill only about one animal in 1,000 million. This number is large compared with the population which is likely to be exposed to the drug.

The following mortalities were observed after the intravenous injection of a solution of neoarsphenamine in mice (Perry, 1950):

0.3 ml. 6/20=30% 0.36 ml. 12/20=60%

From these results it may be estimated that

$$m = \log \text{LD50} = \bar{1}.530$$

$$\lambda = \text{S.D. log tolerance} = 0.1018$$

$$\text{Log safe dose} = m - 6\lambda = \bar{1}.530 - 6 \times 0.1018 = \bar{2}.919$$

and the safe dose is therefore estimated as 0.083 ml. The validity of this extrapolation may be questioned, but it is probably more reliable than an arbitrary guess of the factor of safety.

When some animals die in control groups without drugs allowance can be made for this fact by using Abbott's correction (Gaddum, 1953).

The object of most toxicity tests is not only to save life, but also to avoid minor degrees of injury. It may be possible sometimes to decide which animals have suffered minor damage and to observe an increase in the percentage harmed when the dose increases. When this can be done the method outlined above may be used, but it is not easy when the animals do not die. In experiments of this type observations are usually made of the weight of the animals, and various other measurements may be made such as estimates of food and water consumed, blood pressure, blood counts, temperature, etc. When this has been done it is not immediately obvious how the results may be used to calculate a safe dose which will affect only a negligible proportion of the animals.

Measurements of this kind may diminish as the dose increases, but it is convenient to consider the case where the slope of the dose-effect curve is positive. The effect may, for example, be the difference between the weights of treated and control groups. This is likely to increase as the dose increases. Let x be the logarithm of the dose and y the corresponding measurement (the " effect "). Let s be the estimated standard deviation of the measurements. Consider the case where y is linearly related to x, and the measurements are normally distributed, and the standard deviation of y (σ) is independent of the dose (i.e., the measurements are homoscedastic). If these conditions are not fulfilled, it may be possible to replace the original measurements with some suitable metameter which fulfils them.

It should then be possible to express the results as shown in Fig. 1, where the vertical scale represents the effect (y) and the horizontal scale represents log dose (x). The relation between dose and effect is given by the line CD, the slope of which is b_e. The line OA represents no effect at all—that is the measurement when no dose has been given. Suppose that the results are converted into quantal results by means of the convention that effects larger than EF are positive and smaller effects negative. The percentage of positive effects at the dose DF can be estimated from the deviation DF. Let $DF=k\sigma$; then the expected mortality is that corresponding to a normal equivalent deviation (NED) of k. The relation between dose and mortality can be shown by plotting k against log dose. The slope of

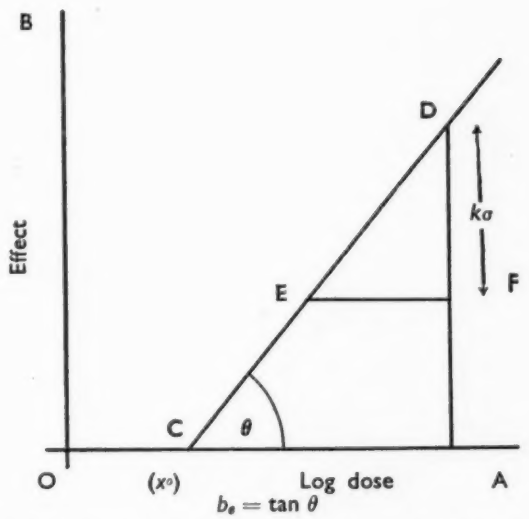

FIG. 1 —Theoretical relation between log dose and effect (see text).

the line relating NED (or probit) to log dose $=b_e=k/EF=1/\lambda$, where λ is the standard deviation of the logarithms of the individual effective doses. This relation between λ and the slope is implicit in the theory of probits. It follows that $EF=k\lambda$. The slope of the line relating effect to log dose $=b_e=DF/EF=k\sigma/k\lambda=\sigma/\lambda \therefore \lambda=\sigma/b_e$. λ can thus be estimated as s/b. This method of calculation has been in use for some time (Gaddum, 1931), but the above argument in support of it has not been given before.

The effect on a single animal is significant when $y=ts$, where t has the appropriate value obtained from tables. For the calculations described here t is taken as equal to 2. This may be called the " threshold effect " and is presumably harmless, since it corresponds to a value of the measurement on which the experiment is based, which is exceeded about once in every 20 of the control animals. The corresponding log dose (x_1) may be called the threshold log dose. It is proposed that the safe dose be calculated from the expression $(x_1-6\lambda)$, which is comparable with the expression $(m-6\lambda)$ used above to calculate the dose which causes a negligible mortality. In both cases, the logarithmic factor of safety is 6λ; in one case it is applied to the LD50 and in the other it is applied to the threshold dose. In practice the safe dose can be estimated as $(x_0-4\lambda)$ where x_0 is the log dose corresponding to no effect and is represented by the point C in Fig. 1.

This argument involves three assumptions which can be tested—that the line is straight and that y is normally distributed and has a constant standard

deviation. It makes no allowance for errors of estimation, but it does provide an estimate, instead of a guess, of the factor of safety.

Example 1: Smith (1950) found that various antimalarials inhibited the growth of young rats and devised a short-term chronic toxicity test in which drugs were mixed with the food for 14 days. The effect of each drug was calculated as the growth suppression (y) which was equal to the difference between the mean weight changes in groups receiving the drug and control groups receiving no drug. When y was plotted against log dose (mg./kg./day) the results could be approximately fitted by straight lines over a certain range of doses in the neighbourhood of the GS50 (the dose causing 50% suppression of growth). Smith gives the standard deviation of the effect for each group and this appears to have been approximately constant for doses less than the GS50. The root mean square of his estimates of the standard deviation of the growth in grams in this range was calculated and taken as an estimate of s. The log dose corresponding to no effect (x_o) was estimated by plotting the actual growth in grams against log dose, fitting a straight line to the results and taking the point on this line corresponding to the growth of the controls. The results did not differ appreciably from the estimates based on the regression line, which were used for the calculations in Table I.

TABLE I

CALCULATION OF SAFE DOSES OF ANTIMALARIALS FROM THE DATA OF SMITH (1950)

	s S.D.(y) g.	b' dy/dx g.	λ s/b'	Safety Factor Anti-log 6λ	Daily Dose (mg./kg.) GS50	Threshold	Safe
Pamaquin hydroiodide	9·87	280	0·035	1·6	30	28	17·3
Quinacrine hydrochloride	8·85	66·5	0·133	6·3	36	13	2·1
Chloroquine diphosphate	6·0	115	0·052	2·0	43	22	11
Chlorguanide hydrochloride	8·5	109	0·078	2·9	71	59	20
Amodiaquine (Camoquin)	5·9	88·4	0·067	2·5	112	78	31
Oxychloroquine diphosphate	7·0	105	0·067	2·5	123	35	13·9
Quinine hydrochloride	7·3	106	0·069	2·6	460	250	96
Thiourea	8·0	22·4	0·357	139	575	160	1·2
Sulphadiazine Sodium	7·6	112	0·068	2·6	1,300	720	290

The value of λ was calculated from Smith's estimates of the slopes of the log-dose-effect lines. His slope function is given as a percentage and was therefore multiplied by the mean weight change of the controls for each drug to give an estimate of the slope (b') in grams. The standard deviation of log tolerance (λ) was then estimated as s/b'. In accordance with the above suggestions the safe dose was estimated from ($x_o - 4\lambda$).

The results of these calculations are shown in Table I. It will be seen that the safety factor was often quite small, but that it was surprisingly high for thiourea because the log-dose-effect curve was very flat ($b' = 22.4$ g.).

Example 2: In the experiments by Barnes and Denz (1954a) drugs were mixed with the diet of rats and the effects of sublethal doses were detected by estimates of cholinesterase in the blood or brain. Groups of 6–12 rats were used for each dose and

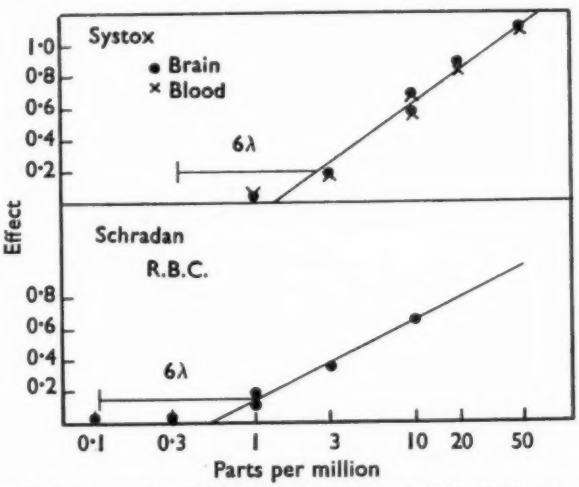

FIG. 2.—Observed relation between the logarithm of the concentration of toxic substances in the diet in parts per million and their effect on cholinesterases, calculated according to the convention described in the text (data of Barnes and Denz, 1954a).

the estimates were made after a period of several weeks on the diet. The cholinesterase was estimated from the rate of production of CO_2 in a Warburg apparatus with acetylcholine as substrate and the results were expressed in μl. CO_2/min./ml. (or g.). The mean result for each group was calculated and in the present calculations its logarithm taken. The difference between this figure and the corresponding figure for control animals in the same experiment was taken as the effect of each dose (y). This was plotted against log dose and the points fitted with straight lines.

The standard deviation of y (s) was estimated from the ranges. This is thought to be justifiable when the number in each group is not more than 12. The maximum and minimum effects in each group of the results, as originally given, were marked and the ratio of one to the other calculated on a

slide rule. The logarithm of this ratio was divided by d_n, which is the expected ratio of the range to the standard deviation.

Table II shows the results with systox (diethyl-*S*-ethylthio-ethanol thiophosphate). The effects on the brain agreed well with the effects on the whole blood and the results could be fitted with a straight line, when y was plotted against log dose.

There was evidence that s tended to increase when the dose was high, and the figures for the highest two doses were therefore omitted in the calculation of the mean values of s. The results for

TABLE II

THE EFFECTS OF DIETARY SYSTOX ON THE CHOLINESTERASES] OF RATS, CALCULATED ACCORDING TO THE CONVENTION DESCRIBED IN THE TEXT

	Dose p.p.m.	Degrees of Freedom	y Effect		s Standard Deviation of y	
			Brain	Blood	Brain	Blood
A	0	6	0	0	0·076	0·080
	1	6	0·033	0·045	0·045	0·065
	3	6	0·181	0·161	0·040	0·063
	10	7	0·697	0·671	0·041	0·077
B	0	11	0	0	0·063	0·103
	10	11	0·580	0·546	0·118	0·193
	20	(10)	0·882	0·832	(0·186)	(0·194)
	50	(11)	1·076	1·080	(3·21)	(4·04)
Total ..		47	Mean ..		0·064	0·097

blood appeared to vary significantly more than those for brain. The higher figure (0.097) was therefore taken as an estimate of s. The threshold effect y_1 (see above)$=2\times0.097=0.194$. This is much smaller than the effect of 20 parts per million (p.p.m.), and this justifies the omission from the calculations of the estimates of s for the highest two doses. The argument is based on the bottom part of the curve only. The following figures were estimated from the regression line:

$$b'=0.66 \qquad x_1=0.414$$

From these figures it may be calculated that

$$\lambda=s/b'=0.097/0.66=0.147$$

The safe log dose$=x_1-6\lambda=0.414-0.832=\bar{1}.532$

The safe dose is thus estimated as about 0.34 p.p.m. It will be seen that the safety factor by which the threshold dose is divided to find the safe dose= antilog $6\lambda=7.6$.

Example 3: Schradan (OMPA, tetrax, octamethylpyrophosphoramide).

When the results were calculated as in Example 2, they could again be fitted by straight lines, but the lines for different tissues did not coincide. The red blood corpuscles appeared to be the most sensitive tissue and then the blood, plasma and brain, in that order. The following calculations are based on the results with the most sensitive tissue, the red blood corpuscles.

It was calculated that $b'=0.458 \quad x_1=0.01$

Therefore $\lambda=s/b'=0.08/0.458=0.175$

Log safe dose$=x_1-6\lambda=0.01-1.05=\bar{2}.96$. Safe dose $=0.09$ p.p.m.

The factor of safety is in this case 11.2.

The results obtained by graphical methods (Fig. 2), in Examples 2 and 3, differed by less than 10% from the results recorded above. Such methods are presumably accurate enough for this type of calculation.

TABLE III

THE EFFECTS OF SCHRADAN IN THE DIET OF RATS ON THE CHOLINESTERASES IN THE RED BLOOD CORPUSCLES

	Dose p.p.m.	Degrees of Freedom	y Effect	s Standard Deviation
A	0	11	0	0·071
	0·1	11	0·03	0·0785
	0·3	11	0·03	0·0693
	1	11	0·19	0·048
B	0	5	0	0·044
	1	5	0·12	0·108
	3	5	0·34	0·150
	10	4	0·63	0·071
Total ..		64	Mean ..	0·080

DISCUSSION

Although it is hoped that the methods described here will help those responsible for chronic toxicity tests, it is clear that many difficulties remain. For example, no allowance has been made for errors in the estimation of m, x_1 and λ.

It was hoped at one time that sublethal doses of drugs would generally inhibit the growth of young animals as they did in the experiments of Smith with antimalarials. This is not, however, a universal rule and the methods of calculation discussed above sometimes cannot be applied unless other methods of measuring sublethal toxic effects are used. The development of new methods of detecting small effects of drugs on the central nervous system may perhaps widen the scope of these methods. These difficulties may be overcome, but a more serious difficulty lies in the fact that results obtained with

animals cannot safely be applied to man. The best that can be done is to test each drug on several different species of animal and to assume that man is no more affected than the most sensitive of the species tested.

Summary

Methods are described for obtaining from the results of a toxicity test a rough estimate of the largest dose which can be considered safe.

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EXPERIMENTS WITH PERFUSED FROG'S SPINAL CORD

BY

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(RECEIVED DECEMBER 1, 1955)

The study of the effects of drugs on spinal reflexes and the identification of substances liberated during nervous activity are of interest in connexion with the hypothesis of the chemical transmission of nervous impulses. The object of the present work was to study the effects of drugs on spinal reflexes in the frog, and to try to detect the liberation of active substances during activity.

The method described by Bunzl, Burgen, Burns, Pedley, and Terroux (1954) has been found suitable, with some modifications, for this investigation. In this method gaseous oxygen is perfused through the blood vessels in order to supply oxygen to the spinal cord, and Ringer solution containing glucose is allowed to run through the vertebral canal. Drugs which are added to the Ringer solution then act directly on the spinal cord without interference from other tissues, and substances liberated at synapses in the cord may appear in the fluid perfusing the vertebral canal without being exposed to possible destruction by blood.

METHODS

Dissection.—Rana temporaria was used. The spinal cord was divided in the brachial region and the portion of the central nervous system above this was destroyed. The body cavity was opened and ligatures were tied around the base of each lung and around the confluence of the two systemic arches to form the aorta. General circulation was thus reduced to the branches coming from the systemic arches, some of which run to the spinal cord. Immediately after, a cannula made from an injection needle and connected to the oxygen supply was tied into the truncus arteriosus and gaseous perfusion was started. The gas was allowed to escape from a slit made in the sinus venosus. A constant pressure of 120 mm. Hg always gave satisfactory results. Bunzl *et al.* found that ligation of the systemic arches above the abdominal aorta excluded the posterior parts of the frog from the general circulation. With the frogs used here it was found that when this was done oxygen passed into the femoral arteries so that the muscle, the contraction of which was recorded, was supplied with oxygen. This did not appear, however, to be essential, since satisfactory results were also obtained when this was prevented by ligation of the femoral artery. After gaseous perfusion had been started the frog was laid prone and the *biceps femoris* was freed from its fascia, blood vessels, and sciatic nerve. The tendon was tied close to its insertion at the knee and cut between the thread and the insertion. When dissection was completed the thread, passing under a pulley, was connected to a writing lever, to give a tension of 2 g. Contractions were isotonic. A cannula made from an injection needle was then pushed into the vertebral canal through the gap made by flexing the urostyle-vertebral-column joint. The insertion into the vertebral canal of the cannula, if not properly done, can cause lesions of the spinal cord or nerves, after which intense spontaneous activity follows. The whole body of the frog was kept moist by a very fine spray of Ringer fluid with no dextrose.

Perfusion of the Vertebral Canal.—The technique used was different from that of Bunzl *et al.* Fig. 1 shows our apparatus : fluid from the reservoir *A* runs into the tube *B* and the rate of flow is controlled with a screw clip so as to maintain a slow overflow through b_1. The tube *B* is marked at b_3 corresponding to 1 ml. below the level of the overflow, so that it is possible to calculate the velocity of flow by stopping the flow from the reservoir and timing the fall of the level of the fluid. The tube b_2 can be opened by a clamp, so that the tube *B* can be emptied rapidly. The tube *c* makes it possible to collect fluid from the tube *B*. The tube *B* is connected with the cannula *D* which has a diameter of about 1 mm. and lies in the lower part of the vertebral canal. Flow in *D* and consequently in the vertebral canal is controlled by a screw clip with a very fine thread. With this apparatus changes of pressure are negligible and the rate of flow is sufficiently constant. The composition of the Ringer is that indicated by Bunzl *et al.*: NaCl 6.5 g., $CaCl_2$ 0.12 g., KCl 0.14 g., dextrose 2 g., and 30 ml. of 0.15 M-sodium phosphate buffer (*p*H 7.4) in a litre.

Stimulation.—With the apparatus described by Bunzl *et al.* two difficulties were encountered : (a) reflex activity could be obtained only for a comparatively short time ; (b) the electric stimuli were apt to escape and cause contractions of the muscles which were not due to reflexes. Bunzl *et al.* used two stimulators delivering alternating current (frequency

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60 c/sec., voltage 0–20). The apparatus used here consisted of two completely independent neon stimulators in each of which the current was derived from dry batteries. The frequency and strength of the stimuli were controlled by suitable resistances and a neon lamp. The connexion between the stimulators and each electrode was through a condenser (0.1 μF). The positive electrode consisted of a piece of platinum foil (1 cm. × 1 cm.) placed under the leg of the frog. The negative electrode, consisting of a spiral of silver wire on which chloride had been deposited, was placed on the foot. With this arrangement the flow of current was concentrated on the foot. In order to avoid loss of current to earth through the body of the frog, the table on which the frog was placed was supported on plexiglass. The right hind foot was stimulated at regular intervals of 30–60 sec. by volleys of stimuli lasting 1 sec. under the control of an electric clock (Palmer). The frequency was 10–15/sec. and the voltage was adjusted to give a suitable response. This stimulus caused a flexion reflex of the right leg; the contractions of the right *biceps femoris* were recorded. In some experiments, in order to study spinal inhibition, alternate stimuli were combined with stimuli on the left hind foot. This stimulation, contralateral to the side of recording, started 1 sec. before the homolateral stimulation and stopped at the same time, so that its duration was 2 sec. The normal effect of this contralateral stimulus was to diminish the effect of the homolateral stimulus, so that in the records responses to homolateral stimulation (uninhibited) alternate with smaller (inhibited) responses due to the stimulation of both feet. When stimuli were submaximal, reflex responses were regular for 6–8 hr., during which the size of the response diminished slowly. Fig. 2 shows constant responses during 4 hr. in an experiment where no drugs were used. When attempts were made to detect active substances in the perfusate both hind feet were stimulated together.

Collection of the Perfusion Fluid.—Fluid was collected by a fine tube lying loosely in the upper end of the vertebral canal, so as not to interfere with the rate

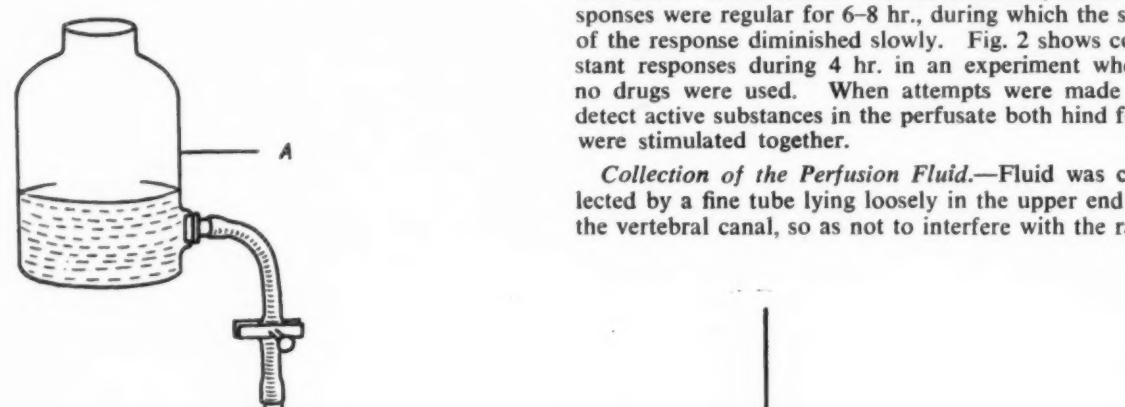

FIG. 1.—A diagram of the apparatus (see text).

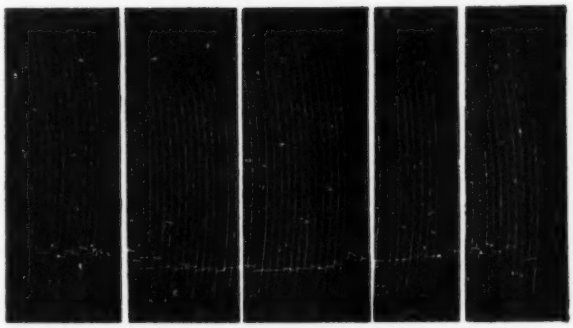

FIG. 2.—Flexor reflex. Interval between stimuli 1 min. Interval between sections of the record 1 hr. Flow 6 ml./hr. Regular responses for 4 hr.

of flow. The tube was connected to a small measuring cylinder to which a negative pressure was applied. This cylinder was immersed in a mixture of ice and water and, as soon as collection was complete, it was frozen in order to preserve active substances for testing later. When drugs were to be tested the velocity of flow was 6–20 ml./hr. When perfusates were to be tested for released substances the flow was about 1–2 ml./hr., but at the beginning of an experiment the perfusion was allowed to proceed for 30–60 min. at a higher velocity in order to wash out tissue debris and remove active substances liberated during dissection. When the perfusate was to be tested for acetylcholine, the perfusion fluid generally contained eserine (8×10^{-6}). Fluid was collected for 1 hr. without stimulation (control period). Stimulation was then applied for 1 hr. and more fluid collected. Sometimes these periods of control and stimulation were repeated.

Drugs.—Doses of drugs are given as the weights of the salts, except for 5-hydroxytryptamine, the dose of which is given as the weight of the base. Concentrations refer to the final concentrations in the perfusion fluid. After addition of the drug to the fluid the *p*H was adjusted, if necessary, to 7.4. Drugs were applied until effects were observed and then washed out again. The following drugs were used : acetylcholine hydrochloride (Roche Products), benzoquinonium ("mytolon," Sterling Winthrop), hexamethonium hydrobromide (May and Baker), 5-hydroxytryptamine creatinine sulphate (Abbott Laboratories), lysergic acid diethylamide (Sandoz Products), reserpine (Ciba Laboratories), substance P_4 (Amin, Crawford and Gaddum, 1954), adrenaline hydrochloride, atropine sulphate, chlorpromazine, carbachol hydrochloride, eserine sulphate, histamine hydrochloride, morphine sulphate, nicotine tartrate, noradrenaline hydrochloride, (+)-tubocurarine chloride.

Spinal Cord Extracts.—The frog was frozen, the spinal cord then dissected out, freed of the roots of the nerves, weighed and crushed in a test tube with a known volume of Ringer solution. The supernatant fluid was used for tests. When tests for acetylcholine were to be made, eserine (8×10^{-6}) was run through the vertebral canal before dissection and used for the extraction.

Test Objects

Leech.—Dorsal muscles from leeches were suspended in 2 ml. Ringer solution. The tension was about 5 g. Acetylcholine was used as a control drug and was diluted shortly before use, from a stock solution kept at *p*H 4 in ice. Solutions were tested every 10 min. and allowed to act for 3 min.

Heart of Spisula solida.—The technique used was that described by Gaddum and Paasonen (1955). The heart was suspended in 1–2 ml. of a solution of the following composition : NaCl, 23 ; Na_2SO_4, 4 ; KCl, 0.65 ; $CaCl_2$, 1.1 ; $MgCl_2.6H_2O$, 10 ; $NaHCO_3$, 0.2 g./l. Drugs were allowed to act for 30 sec. and HT was used as a standard. Benzoquinonium (10^{-5}) was sometimes present in the solution as an antagonist to acetylcholine.

Guinea-pig's Intestine was suspended in 2 ml. Tyrode solution at 37°. Drugs were allowed to act for 30 sec. Carbachol and histamine were used as control drugs.

Rat's Uterus was prepared by subcutaneous injection of stilboestrol (10 μg./100 g.) into the rat on the day before the experiment. The uterus was suspended in 2 ml. of Jalon's solution at 30°. Drugs were added in small volumes and allowed to act for 30 sec. ; consistent results were obtained in several experiments with each drug.

RESULTS

Effects of Drugs on the Spinal Reflexes

Preliminary experiments showed that the reflexes were reversibly depressed by ether, chlorbutol and anoxia.

Ether was dissolved in the perfusion liquid or vaporized in the immediate neighbourhood of the

FIG. 3.—Flexor reflex. Interval between stimuli 1 min. Flow 12 ml./hr. Between arrows chlorbutol 10^{-4} for 5 min. (twice in the same experiment). Depression of flexor reflex followed by recovery.

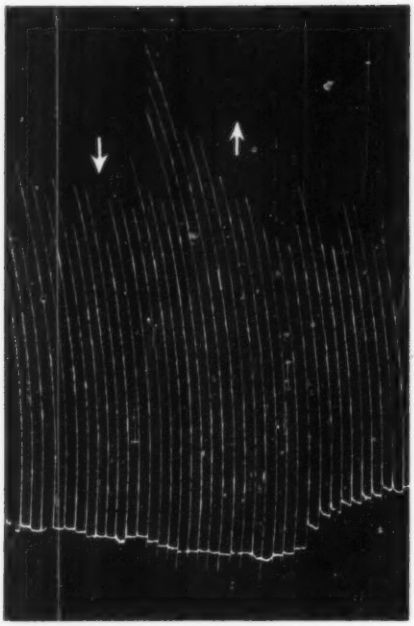

FIG. 4.—Flexor reflex. Interval between stimuli 1 min. Flow 6 ml./hr. Between arrows acetylcholine 3×10^{-4}. Temporary increase of response.

frog. In the latter case the circulation was intact but the brain destroyed. The effect of ether appeared quickly: after a brief period of excitation in which reflex responses were bigger and accompanied by spontaneous contractions, the ether caused progressive reduction of the responses, both inhibited and uninhibited. The degree of depression and the duration of the effect depended on the duration of exposure. At the end of the period of administration the reflexes recovered slowly, but the rate of recovery could be increased by the administration of oxygen. The recovery was complete for both inhibited and uninhibited responses.

Chlorbutol in a concentration of 10^{-4} in the perfusion fluid caused depression of the flexor reflex. As shown by Fig. 3, the effect appeared almost immediately and reflexes were quickly abolished. At the end of the period of administration reflexes quickly recovered.

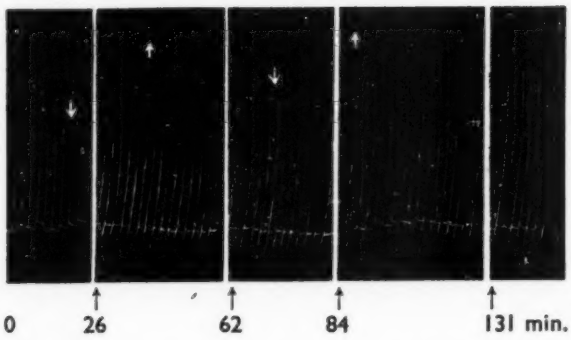

FIG. 5.—Flexor reflex. Interval between stimuli 1 min. Flow 15 ml./hr. Between arrows nicotine tartrate 10^{-4} from 7 to 32 min. and from 67 to 85 min. Delayed increase of the response.

Anoxia due to stoppage of the flow of oxygen caused depression and finally disappearance of both reflexes, inhibited and uninhibited. After periods of 7 min. of anoxia, recovery was complete in 1 hr. It appeared that reflexes could recover even after 15–20 min. of anoxia.

Acetylcholine in a concentration of 10^{-6}–10^{-4} in the perfusion fluid had usually no effect on the reflexes, even when it was accompanied by eserine (5×10^{-5}). High concentrations of ACh ($>10^{-4}$) caused an increase in the reflex response. The effect appeared quickly and was generally of very short duration; in Fig. 4 the effect of ACh is vanishing before the administration is stopped. Sometimes still higher doses appeared to have an inhibitory action.

Nicotine in a concentration of 10^{-4} increased the reflexes, as shown by Fig. 5. At the end of administration recovery was complete in 1 hr. In Fig. 5 a subsequent dose caused a larger response; with higher concentrations there was depression of the flexor reflex.

Mephenesin at a concentration of 2×10^{-5} caused progressive depression of the reflexes or complete suppression. When the drug was removed recovery was complete. After a higher concentration (10^{-4}) recovery was not possible.

Chlorpromazine in a concentration of 10^{-4} depressed the reflexes; when the drug was

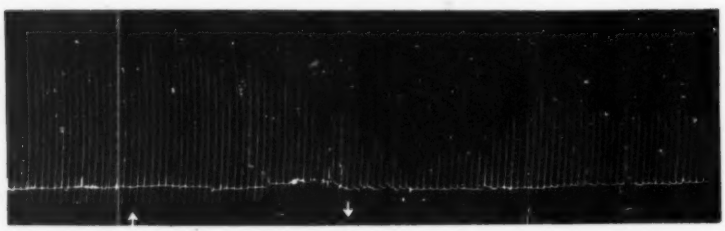

FIG. 6.—Flexor reflex. Interval between stimuli 1 min. Flow 10 ml./hr. Between arrows chlorpromazine 10^{-4}. Depression followed by recovery.

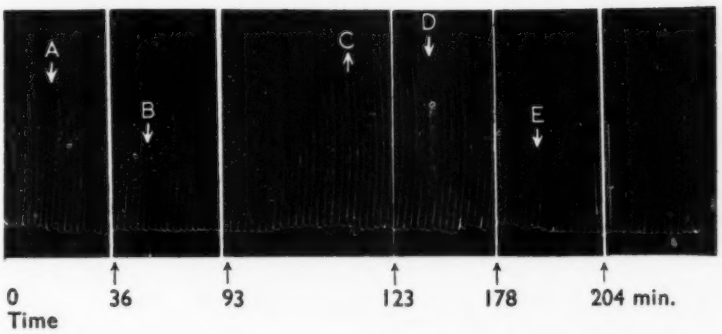

FIG. 7.—Flexor reflex. Interval between stimuli 1 min. 7.5 ml./hr. A–B, reserpine 10^{-7}; depression. B–C, reserpine 10^{-7}+LSD 10^{-6}; recovery after about 1 hr. C–D, no drugs. D–E, reserpine 10^{-7}; depression. E–F, reserpine 10^{-7}+LSD 10^{-6}; no recovery.

removed recovery occurred (Fig. 6). Both the effect and recovery appeared fairly quickly.

Reserpine in a concentration of 10^{-7} depressed or completely abolished the reflexes. There was always a latent period of 30–90 min. The depression of the reflexes was irreversible even after many hours.

Lysergic acid diethylamide in a concentration of 10^{-6} added to the reserpine was found to arrest the gradual development of depression due to reserpine, and sometimes there was recovery of the response (Fig. 7). In other cases, however, it was impossible to demonstrate any such effect.

5-Hydroxytryptamine in a concentration of 5×10^{-6} did not alter the depressant action of reserpine even when administered for a very long time, as in Fig. 8. Reflexes which had been depressed with reserpine were restored by strychnine (Fig. 8).

Morphine in a concentration of 5×10^{-5} quickly caused slight depression of the direct effect of the homolateral stimulation, and definite depression of the inhibitory effect of stimulation of the contralateral foot. At the end of the period of administration reflexes recovered completely (Fig. 9). The depressant effect was obtained more than once in the same preparation, but the spinal cord appeared to become insensitive to the morphine after several doses.

Strychnine in a concentration of 5×10^{-5} first increased the reflex response to homolateral stimulation and abolished the inhibitory effect of contralateral stimulation. The response to homolateral

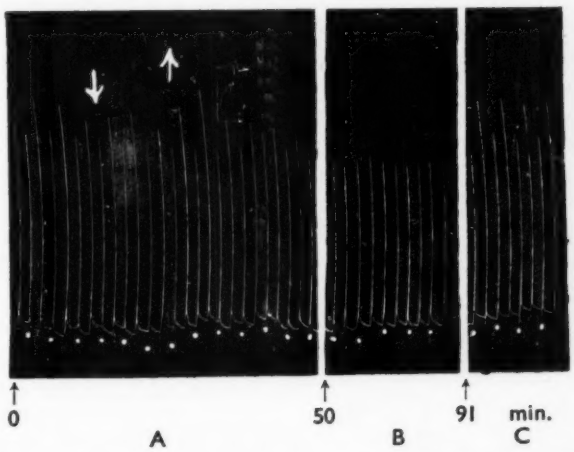

FIG. 9.—Flexor reflex. Interval between stimuli 1 min. At white dots simultaneous stimulation of both feet inhibits response. A—Between arrows morphine 5×10^{-5}; B—depression of both reflexes, especially of the inhibitory one; C—recovery.

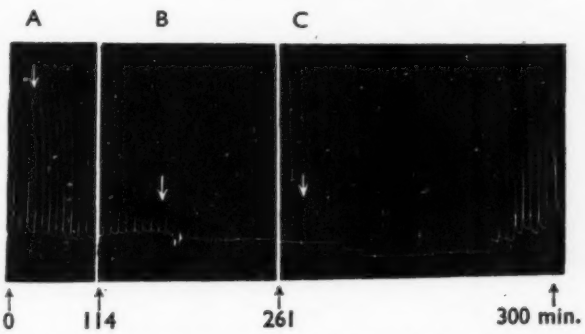

FIG. 8.—Flexor reflex. Interval between stimuli 1 min. Flow 6 ml./hr. A–B, reserpine 10^{-6}; depression. B–C, HT 10^{-5}; no recovery. C-onwards, strychnine 10^{-6}; recovery after 26 min.

stimulation then became smaller than the response to bilateral stimulation. When the response was recorded on a fast drum it was seen that contralateral stimulation actually caused contraction of the muscle in the fraction of time (1 sec.) by which contralateral stimulation preceded homolateral stimulation. The contralateral stimulation before strychnine caused a small relaxation of the muscle or no effect at all, except a diminution of the response to homolateral stimulation. In Fig. 10 it will be seen that the response to bilateral stimulation which had originally been less than that to homolateral stimulation became greater after strychnine. The effect of strychnine is slow to appear (generally 30 min.) and to develop to the

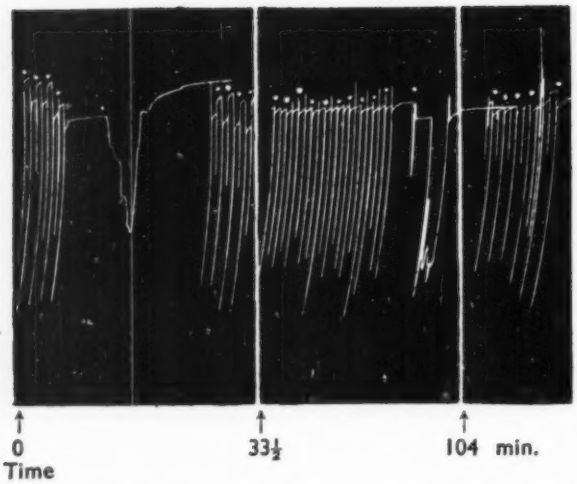

FIG. 10.—Flexor reflex. Interval between stimuli 30 sec. Flow 6 ml./hr. At white dots simultaneous stimulation of both feet before strychnine inhibits response. After strychnine this stimulation increases the response. At 4.5 min. and 104 min. the drum was run quickly for a short time.

stage of complete ineffectiveness of inhibitory stimuli.

No effect on flexor reflexes, uninhibited or inhibited, was demonstrated for *adrenaline, noradrenaline, carbachol, atropine, eserine, hexamethonium, tubocurarine, histamine, 5-hydroxytryptamine, substance P,* or *benzoquinonium,* in concentrations of these substances up to 10^{-4}.

Active Substances in the Perfusates

Perfusion fluids collected from the vertebral canal have been shown to produce effects on leech muscle, *Spisula* heart, guinea-pig ileum and rat uterus. The activity was always comparatively high in fluids collected during the first hour after dissection, whether the reflexes were stimulated or not; it usually fell to a minimum in 3–4 hr. and then remained stable if no stimuli were applied. Crude extracts of the spinal cord had similar effects.

Leech Muscle.—In most experiments eserine (8×10^{-6}) was present both in the perfusion fluid while it was in contact with the spinal cord, and in the fluid bathing the leech muscle. Such perfusion fluids usually caused a contraction of the muscle (Fig. 11). This was compared with that due to acetylcholine, and the apparent concentration of this substance is called the ACh-equivalent (Chang and Gaddum, 1933). Such effects were not observed when the leech had not been treated with eserine, but the presence of eserine in the perfusion fluid in contact with the spinal cord did not seem to be essential, since perfusates collected soon after dissection and containing no eserine sometimes caused contractions of the eserinized leech. However, no effects were seen with perfusates collected in later periods of the experiments in the absence of eserine in the perfusion fluid.

TABLE I

ACETYLCHOLINE-EQUIVALENT IN PERFUSATES COLLECTED IN DIFFERENT EXPERIMENTS

BS, before stimulation; DS, during stimulation; AS, after stimulation; DSA, during spontaneous activity.

Date	Perfusate	ng./ml.
Aug. 26	DSA	22
„ 27	BS	13
	DS	33
„ 30 .. .	DSA	11
Sept. 1	BS	8
	DS	11
	AS	7
„ 2	BS	2
	DS I	4
	AS	<1
	DS II	6
„ 3	BS	2
	DS I	4
	DS II	7
„ 5	BS	—
	DS I	—
	AS I	2
	DS II	4
	AS II	<1
	DS III	1

The results are shown in Table I. In the periods of reflex activity due to stimulation of the feet the ACh-equivalent of the perfused fluid increased; and during the next hour it generally fell to values below those observed before stimulation. In some

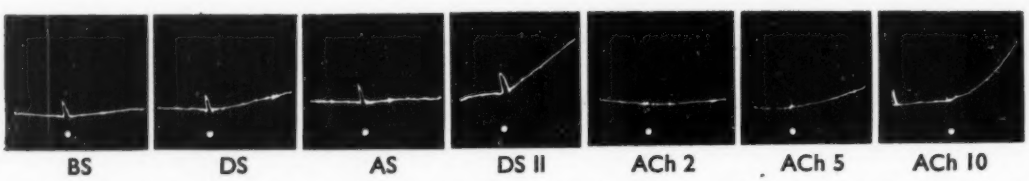

FIG. 11.—Leech muscle (Sept. 3). Eserine 8×10^{-6}. Undiluted perfusate applied to the leech. BS, before stimulation; DS, during stimulation; AS, after stimulation; DS II, during second period of stimulation. Acetylcholine (ACh) doses in nanograms in 1.5 ml.

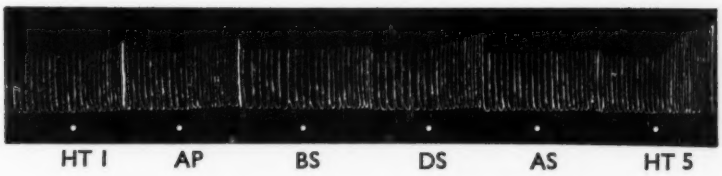

FIG. 12.—*Spisula* heart in 2 ml. bath (Sept. 5). 0.2 ml. of perfusates added to the bath. AP, after pithing; BS, before stimulation; DS, during stimulation; AS, after stimulation. HT doses in nanograms in bath.

experiments the ACh-equivalent during reflex activity was higher than that during the first hour after dissection. Generally it was lower than this, but when sufficient time was allowed after dissection the ACh-equivalent of the fluid collected during stimulation was always greater than those of control fluids, collected just before and after the stimulation. In two experiments a second period of stimulation caused a second rise of the ACh-equivalents (Sept. 2 and Sept. 5). In several experiments intense spontaneous activity of the frog was found to be associated with high ACh-equivalents (Aug. 26 and Aug. 30).

Spisula solida *Heart.*—Perfusates caused an increase of the amplitude and frequency of the beat (Fig. 12). Perfusates collected during the first hour after dissection, or during spontaneous activity of the frog, were more active than those collected later, even if the reflexes were stimulated. On the other hand, when sufficient time was allowed for the activity to fall to low values, stimulation appeared to increase the activity. The effects were compared with those of 5-hydroxytryptamine, and the HT-equivalents are shown in Table II.

In one experiment the HT-equivalent of a perfusate was estimated as 15 ng./ml. on the heart of *Spisula* and 15 ng./ml. on the rat uterus.

Since reserpine is known to liberate 5-hydroxytryptamine (Pletscher, Shore, and Brodie, 1956), experiments were made with this substance. Control samples were first collected during stimulation in the absence of reserpine and compared with samples collected later for 1 hr. during stimulation while reserpine (10^{-6} to 10^{-7}) was present in the perfusion fluid. Contrary to expectation the perfusates which contained reserpine had less effect on the heart than the control samples (Fig. 13). These concentrations of reserpine did not alter the response of the heart to known amounts of 5-hydroxytryptamine, so that the results suggest that in these conditions reserpine actually inhibited the release of 5-hydroxytryptamine-like substance from the spinal cord.

TABLE II

5-HYDROXYTRYPTAMINE-EQUIVALENT IN PERFUSATES COLLECTED IN DIFFERENT EXPERIMENTS

BS, before stimulation; DS, during stimulation; AS, after stimulation; DSA, during spontaneous activity; ^{0}, in this second hour the spontaneous activity was less intense; ^{00}, in this second hour the intensity of stimulation was greater. In some assays benzoquinonium (10^{-7}) was present in the bath.

Time from the End of Dissection (hr.)	Date	Perfusate	HT-equivalent (ng./ml.)	
			Benzoquinonium Absent	Present
0	Aug. 26	Washing	—	—
0·5		DSA	59	—
1·5		DS	47	—
2·5		AS	4	—
0	,, 29	Washing	—	—
0·5		DSA	47	—
1·5		DSA^{0}	37	—
2·5		DS I	10	—
3·5		DS II^{00}	23	—
0	Sept. 3	Washing	—	—
0·5		BS	>25	>25
1·5		DS I	>25	9
2·5		AS	11	4
3·5		DS II	12	16
0	,, 5	Washing	—	—
1		BS	12	9
2		DS I	7	6
3		AS I	4	5
4		DS II	20	13
5		AS II	6	4
6		DS III	—	—
0	,, 7	Washing	—	—
1		BS	6	—
2		DS	17	—
3		AS	11	9
4		DS II	14	12
0	,, 8	Washing	—	—
1		BS I	20	—
2		BS II	4	—
3		DS	9	—
4		AS	16	—

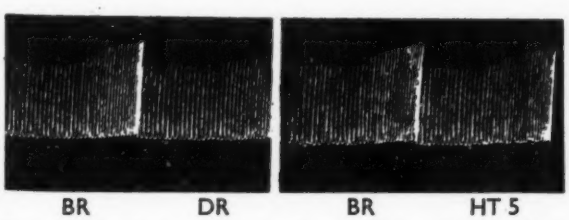

FIG. 13.—*Spisula* heart in 2 ml. bath. 0.2 ml. of the perfusates added to the bath. All samples collected during stimulation. BR, before reserpine; DR, during reserpine 5×10^{-6}. HT doses in nanograms in the bath.

Guinea-pig intestine usually contracted when 0.2 ml. of a perfusate was added to the bath (Fig. 14). Fluids collected during stimulation generally had more effect than fluids collected before or after stimulation (controls), but sometimes responses

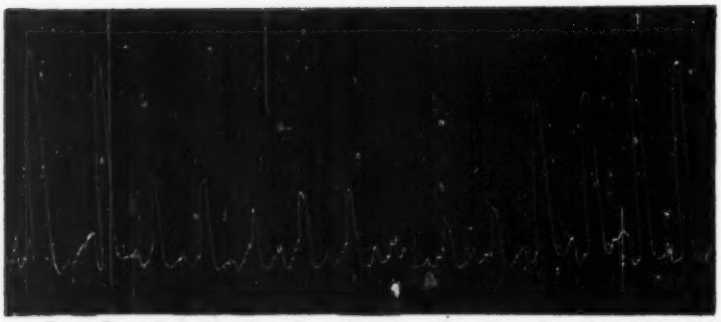

FIG. 14.—Guinea-pig ileum in 2 ml. bath. 0.2 ml. of the perfusates added to the bath. B_1–B_4, successive samples before stimulation (30 min. each); S, during stimulation sample (30 min.). C, carbachol doses in nanograms in bath.

were irregular and obscured by the delayed effects of previous applications of the perfusates, and no clear evidence could be obtained.

The response was not altered by mepyramine (10^{-6}), but atropine had a variable effect upon it. In some experiments it was unaffected by a concentration of 10^{-7}, and in others it was almost completely inhibited by a concentration of 10^{-9}. The concentration causing complete inhibition was generally 5–10 times that which would just cause no inhibition. Concentrations of atropine just below those which inhibited the response to perfusates caused a partial inhibition of the response to equivalent doses of 5-hydroxytryptamine (25–50 ng./ml.), but did not alter the response to equivalent doses of substance P (0.25–0.5 U./ml.). Slightly higher concentrations diminished the responses both to the perfusates and to a preparation of substance P.

Rat uterus usually contracted when 0.1 to 0.2 ml. of a perfusate was added to the bath. The response was not altered by mepyramine (10^{-6}) or atropine (10^{-7}). Few experiments were made and no evidence was obtained of any difference between perfusates collected during stimulation and control perfusates.

DISCUSSION

Evidence for chemical transmission in the central nervous system can be obtained by investigating (a) the effects of drugs on the C.N.S. and (b) the liberation of substances during activity of the C.N.S.

The Effects of Drugs on the C.N.S.

The value of experiments on the effects of drugs by the method described here is limited by our ignorance of the factors which determine whether the drugs present in the perfusion fluid can pass through the meninges and reach their site of action. The technique used for stimulating the reflexes and recording the responses is, of course, crude compared with some of the methods used by electrophysiologists. Moreover, by this technique it is only possible to study the polysynaptic flexor reflex ; it is not directly applicable to monosynaptic reflexes. In the light of these considerations and of the fact that only frogs were used, negative and uncertain results can have only a limited significance, particularly when the drugs used are known to act on the central nervous system. For example, adrenaline has been found to increase flexor reflexes (Bülbring and Burn, 1941) and choline esters increase the height and duration of action potentials in the spinal roots (Malcolm and Wurzel, 1955).

According to Häusler and Sterz (1952) histamine stimulates reflexes in the perfused spinal cord of a frog. This result has not been confirmed by the present experiments.

The absence of results with noradrenaline, atropine, hexamethonium, 5-hydroxytryptamine, substance P and benzoquinonium does not mean that these drugs have no actions on reflexes.

The action of eserine on the spinal cord is important because it provides indirect evidence for or against the presence of cholinergic nerves. This action has been demonstrated by many workers (for literature before 1950 concerning acetylcholine and the nervous system, see Feldberg's reviews, 1945, 1950). According to Wikler (1945) eserine only increases monosynaptic reflexes and has no action on polysynaptic reflexes. When eserine is injected intravenously it increases the sensitivity of the cells of Renshaw to acetylcholine injected intra-arterially (Eccles, Eccles, and Fatt, 1955). In the results recorded here eserine (4×10^{-6}) had no apparent effect on the reflex responses. This negative result has little meaning: we cannot be sure that the eserine reached the nervous tissue in sufficient concentration ; even strychnine did not appear to reach the tissues in less than 20–30 min.

A direct action of acetylcholine on the spinal cord has been recorded in many ways. It has

usually been injected intra-arterially (Feldberg, 1945, 1950; Feldberg, Gray, and Perry, 1952; Marrazzi, 1953; Eccles *et al.*, 1955), but has also been applied locally to the surface of the cord (Feldberg, 1945, 1950) or by micro-injection (Kennard, 1951). In the present experiments it has not always been possible to show an effect, and in any case the doses were rather high—10^{-4} when the rate of flow was 6 ml./hr. in the absence of eserine. However, this concentration is not very different from that used by Eccles *et al.* (1955) to stimulate the cells of Renshaw. The main effect, when present, was an increase of the reflex response. In some cases still higher doses appeared to have an inhibitory action.

There is not much evidence about the action of nicotine on the spinal cord, but its effect appears to be excitation followed by depression. Nicotine excites the cells of Renshaw, but its action differs from that of acetylcholine in not being increased by eserine (Eccles *et al.*, 1955). In small doses nicotine accelerates the response to strychnine (Bonnet and Bremer, 1952). The results shown in Fig. 10 confirm the observation of Bunzl *et al.* (1954) that nicotine increases the response in the flexor reflex of the frog.

Tubocurarine is said only to influence monosynaptic reflexes (Bernhard, Taverner, and Widen, 1951). It had no action on the polysynaptic reflex studied here.

The results with strychnine agree with those of Bradley, Easton, and Eccles (1953) and of Bunzl *et al.* (1954). The inhibitory effect of the contralateral stimulation appeared to be converted into an excitatory effect. This confirms the results of Sherrington (1906), but does not disprove the conclusions of Creed and Hertz (1933) regarding the mechanism of this effect.

Wikler (1945) found that morphine increased monosynaptic reflexes and depressed polysynaptic reflexes. In the present experiments morphine depressed two polysynaptic reflexes—the flexor reflex, and the inhibition of this reflex by contralateral stimulation. Inhibitory reflexes in the spinal cord, but not excitatory reflexes, may develop tolerance for morphine (Wikler and Carter, 1953). Sometimes when the same dose of morphine was repeated, the response of both reflexes to the second dose was weaker and briefer.

Mephenesin had a powerful inhibitory action on the reflexes—as was to be expected, since this drug is known to inhibit polysynaptic but not monosynaptic reflexes (Hennemann, Kaplan, and Unna, 1949).

No published information about the action of chlorpromazine on the spinal cord has been found. The effect was purely depressor.

The actions of reserpine have not yet been systematically studied, but its main action is depression of the central nervous system (Bein, 1953; Symposium, 1954). In the present experiments low concentrations progressively depressed or abolished the reflexes after a long latent period (30–45 min. or more). This depression was reversed by strychnine and sometimes appeared to be antagonized by lysergic acid diethylamide.

The Liberation of Substances During Activity of the C.N.S.

The results of the present experiments appear to show that reflex activity in the frog is associated with the release of at least 3 active substances in the perfusion fluid. These 3 substances were detected by tests which are sensitive to, and fairly specific for, acetylcholine, 5-hydroxytryptamine and substance P, but it has not yet been possible to obtain sufficient evidence to identify any of these substances with certainty.

The effects on the leech may be due to acetylcholine. It is *a priori* possible for this substance to diffuse out of the spinal cord, since it has been found in the cerebrospinal fluid (Feldberg, 1945, 1950) and its concentration may be increased in experimental convulsions (McIlwain, 1955). The activity on the leech muscle detected in our perfusates cannot be attributed with certainty to acetylcholine, since this test is not absolutely specific. The fact that no activity is recorded in the complete absence of eserine suggests a choline ester, but further experiments will be necessary before it is known whether it is acetylcholine or not. The amounts liberated in the early stages after dissection were so large that it was not necessary for eserine to be present in the perfusion fluid. In the later stages no activity was detected unless eserine was present both in the perfusion fluid and in the leech bath. The continuous liberation of small quantities during rest need cause no surprise. The increase in the concentration found during stimulated reflex activity suggests that the release of this substance plays a part in this activity.

The heart of *Spisula solida* is a specific reagent for 5-hydroxytryptamine, and the effects of the perfusates may be due to this substance. The agreement in one experiment of the quantitative determination of the activity of a perfusate on *Spisula* heart and rat uterus provides some evidence that the effects were probably due to 5-hydroxytryptamine. They were certainly not due to acetylcholine, which inhibits this heart. It is unlikely

that they were due to adrenaline, since the heart is about 1,000 times less sensitive to adrenaline than it is to 5-hydroxytryptamine. The results suggest that the liberation of 5-hydroxytryptamine may play a part in spinal reflexes.

There is evidence that reserpine liberates 5-hydroxytryptamine from its natural sites in the body: the intestine (Pletscher, Shore and Brodie, 1956; Erspamer, 1956a and b) and the brain (Pletscher, Shore and Brodie, 1956; Paasonen and Vogt, 1955; Correale, 1956), and the theory has been proposed that the central effects of reserpine are due to this liberated 5-hydroxytryptamine (Shore, Silver and Brodie, 1955a and b). The results recorded here showed, contrary to expectation, that reserpine decreased the amount of the substance active on *Spisula* heart present in the perfused fluid.

The substance present in the perfusates which caused contraction of the guinea-pig ileum was not acetylcholine or histamine, because its action was not inhibited by concentrations of atropine and mepyramine which inhibited the responses to these drugs. The results suggest that it is more likely to have been substance P than 5-hydroxytryptamine, but further work will be needed to identify it with certainty and to decide whether its rate of liberation is increased during reflex activity.

Summary

1. The spinal cords of frogs were perfused by a modification of the method in which gaseous oxygen flows through the blood vessels and Ringer solution flows slowly over the cord. The flexor reflex gave regular responses to electric stimulation of the skin of one foot once a minute for 6–8 hr.

2. The reflex was reversibly depressed by anoxia, ether, chlorbutol, mephenesin, chlorpromazine, reserpine or morphine; it was increased by acetylcholine, nicotine or strychnine. Lysergic acid diethylamide appeared to antagonize reserpine.

3. Electric stimulation of the skin of both feet caused the appearance in the perfusates of substances acting like acetylcholine and 5-hydroxytryptamine on eserinized leech and *Spisula* heart, but the evidence on the nature of these substances is still incomplete.

This research was carried out at the suggestion of Professor J. H. Gaddum, to whom I wish to express my sincere gratitude for all his advice and criticism.

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Brit. J. Pharmacol. (1956), **11**, 171.

INHIBITION OF GASTRIC SECRETION IN RATS BY SOME QUATERNARY DERIVATIVES OF ATROPINE

BY

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(RECEIVED DECEMBER 21, 1955)

The increasing use of quaternary amines as inhibitors of gastric secretion suggested a study of some quaternary derivatives of atropine. Visscher, Seay, Tazelaar, Veldkamp, and Vander Brook (1954) have reported that hyoscine methyl bromide is a potent inhibitor of gastric secretion in rats. The reduction of gastric secretion produced by the synthetic quaternary ammonium compounds methantheline bromide (" Banthine ") and propantheline bromide (" Probanthine ") is said to be due to an atropine-like action—at least in the doses used clinically (Johnson and Wood, 1954).

During the war a study (Ing, Dawes, and Wajda, 1945; Ing, 1946) of synthetic atropine analogues was undertaken, but it did not extend to substances containing the tropine nucleus. This was because the synthesis of atropine described by Robinson (1917) was still impracticable, owing to the cost of one of the starting substances. Newer methods of obtaining one of these substances, succinaldehyde, from furan are now available.

The present work compares the effects of atropine sulphate and some quaternary atropine derivatives on gastric secretion in rats with the pylorus tied. The drugs used were atropine methyl iodide, atropine ethyl bromide, atropine propyl iodide, atropine benzyl chloride, and atropine *p*-nitrobenzyl chloride. Atropine propyl halide and *p*-nitrobenzyl halide do not seem to have been described before. The drugs were kindly supplied by Drs. R. G. Johnston and C. G. Haining, of Messrs. T. & H. Smith, Ltd., Edinburgh.

METHODS

The method used is a modification of that of Visscher *et al.* (1954). Male albino rats (160 to 260 g. body weight) were used. They were all taken from the same colony, as rats of some strains have a poor spontaneous secretion. The animals were housed at 70° F., and fed on a pelleted diet. They were fasted in cages containing groups of six to twelve. The cages had wide-meshed wire bases to minimize coprophagy.

The pre-operative fast from solid food lasted 48 hr. During the first 24 hr. of this period the animals had a choice of two bottle-feeds, one of skimmed fresh milk to which had been added 5% glucose and 0.4% NaCl, the other of water. During the next day, the animals had a choice of 5% glucose, 0.4% NaCl, and plain water. On the night before operation the animals had a choice of three drinks—0.4% NaCl, 0.2% NaCl, and water. The rats were shaved on the day before the fast, and were weighed at the end of the fasting period.

Under methyl-*n*-propyl ether and ethyl ether anaesthesia, a 4 cm. midline abdominal incision was made, and the wound held open with self-retaining eyelid retractors. A ligature was placed around the duodenum near the pylorus, in such a way as to avoid trauma to the vessels. Five ml. of 0.8% NaCl was placed in the peritoneal cavity after the closure of the muscle layer with three silk sutures. The skin wound was closed with Michel clips and painted with collodion. The operation usually lasted 3 min.

The drug, in aqueous solution, was injected intramuscularly or intraduodenally in volumes of 0.1 or 0.2 ml./100 g. body weight. The rats were deprived of water and confined in separate cages during the collection period, which was 3 hr. both with the intramuscular and intraduodenal injections. The animals were then killed with chloroform, the abdomen was opened, and the stomach was removed. Gastric fluid was placed in a graduated centrifuge tube, and the fluid volume measured after centrifugation. Gastric fluid is defined as the total gastric fluid volume. There was sometimes contamination with faeces and blood, and the fluid must consist in part of saliva. Nevertheless, the control fluid had usually a *p*H in the range 1.4 to 2.2.

The volume of the gastric fluid in the treated animals, at several dose levels, was compared with that found in simultaneously operated and sham-injected controls. Groups of at least four rats were used at each dose level ; the mean number of rats at each dose level was about six.

RESULTS

Intramuscular Injections.—The dose-response lines in Fig. 1a and b relate the dose given by intramuscular injection to the mean gastric fluid volume

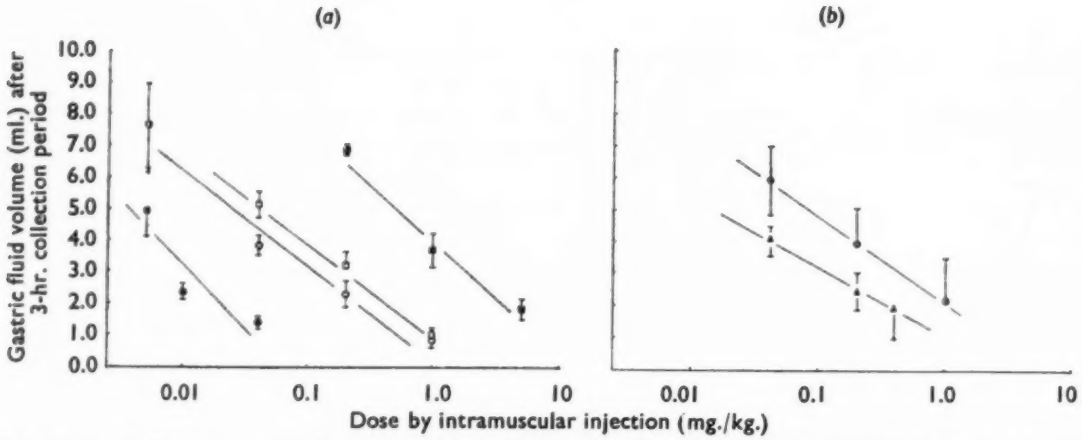

FIG. 1*a* and *b*.—Dose-response relations for inhibition of gastric secretion in rats after intramuscular injection of various atropine derivatives. The lines were drawn by eye. Vertical bars represent the standard errors of the means. Symbols: O, sulphate; ○, benzyl chloride; ●, methyl iodide; ▲, *p*-nitrobenzyl chloride; □, ethyl bromide; ■, propyl iodide.

at the end of a 3 hr. collection period. The vertical bars represent the standard error of the mean. The mean control secretion was 4.8 to 7.3 ml. No correction is made for the differences in molecular weight of the various derivatives.

Approximate ED50 values, calculated from these data, give the relative potencies listed in Table I, where atropine is assigned an arbitrary potency of 1.0. Atropine methyl iodide is the most potent of the drugs tested. Atropine ethyl bromide and atropine *p*-nitrobenzyl chloride are of the same order of activity parenterally as atropine sulphate, and atropine benzyl chloride and atropine propyl iodide are notably less active.

TABLE I

INHIBITION OF GASTRIC SECRETION: APPROXIMATE POTENCY OF QUATERNARY ATROPINE DERIVATIVES BY INTRAMUSCULAR INJECTION

Atropine Derivative	Approx. ED50 (μg./kg.)	Rel. Potency (Atropine = 1·0)
Sulphate	130	1·0
Methyl iodide	12·5	10
Ethyl bromide	220	0·6
Propyl iodide	2,100	0·06
Benzyl chloride	950	0·14
p-Nitrobenzyl chloride ..	300	0·4

Intraduodenal Injections.—The results of the assays in which the substances were injected intraduodenally are indicated in Fig. 2a and b. If Fig. 2b is compared with Figs. 1a and 2a it will be seen that atropine methyl iodide, although exceedingly potent parenterally, is less potent than atropine sulphate by the duodenal route.

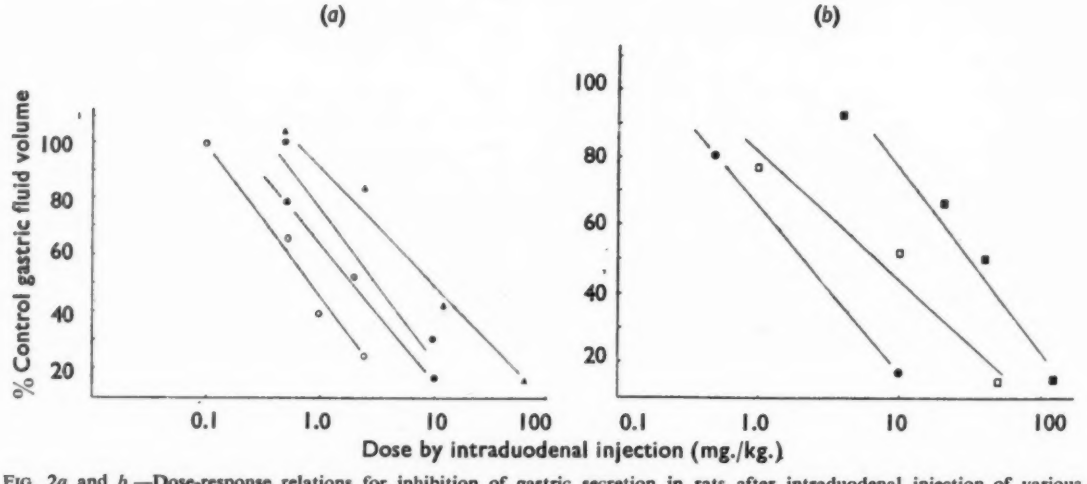

FIG. 2*a* and *b*.—Dose-response relations for inhibition of gastric secretion in rats after intraduodenal injection of various atropine derivatives. Symbols: O, sulphate; ○, benzyl chloride; ●, methyl iodide; ▲, *p*-nitrobenzyl chloride; □, ethyl bromide; ■, propyl iodide.

The third column of Table II shows that the ratio of the intraduodenal ED50 to the intramuscular ED50 for the methyl-, ethyl-, and propyl-quaternary derivatives of atropine is lowest for the propyl derivative and highest for the methyl derivative.

TABLE II

INHIBITION OF GASTRIC SECRETION: APPROXIMATE POTENCY OF QUATERNARY ATROPINE DERIVATIVES BY INTRADUODENAL INJECTION; RATIO OF INTRADUODENAL ED50 TO INTRAMUSCULAR ED50

Atropine Derivative	Approx. ED50 (μg./kg.)	Rel. Potency (Atropine = 1·0)	Duodenal ED50 / i.m. ED50
Sulphate	800	1·0	6·2
Methyl iodide ..	1,900	0·4	160
Ethyl bromide ..	6,500	0·1	30
Propyl iodide ..	26,000	0·03	12
Benzyl chloride ..	3,100	0·25	3·3
p-Nitrobenzyl chloride	9,800	0·1	33

DISCUSSION

The use of a preparation in which the pylorus is ligated, and the animal allowed to recover from the anaesthetic and collect juice in its stomach for a number of hours, derives from the studies of Shay, Komarov, Fels, Meranze, Gruenstein, and Siplet (1946) concerning the production of gastric ulcers in rats. Most of the literature of twenty-odd papers on the method deals with the production of ulcers or with the prevention of ulceration.

The basis of the method is the observation of Shay *et al.* (1946) that substantial quantities of gastric juice are secreted only if the animals are quickly operated on under light anaesthesia and are conscious during the collection period. The rate of secretion under continuous urethane anaesthesia is less than 0.1 ml./100 g. body weight/hr. (Komarov, Shay, Rayport, and Fels, 1944). Inhibition of secretion of juice following pyloric ligation under light barbiturate anaesthesia was used as a measure of enterogastrone activity by Friedman and Sandweiss (1946) and by Visscher (1948). The former used a 90-minute collection period, and the latter two hours: both gave the hormone intravenously.

Atropine and methantheline were assayed by Visscher and Tazelaar (1951) in rats in which the pylorus had been ligated under ether. The drugs were injected intravenously, and a two-hour collection period was used. In the study of hyoscine methyl bromide by Visscher *et al.* (1954), the drugs were given intravenously with collection of juice in two hours, and intraduodenally with collection of juice in three hours. After intravenous injection the action lasted only two hours, so that a longer collection period could not be used.

In the present work, the parenteral assay involved the intramuscular injection of the drug. This gives for atropine an ED50 comparable to that obtained by Visscher *et al.* (1954) by the intravenous method, and admits of the use of a three-hour collection period. Thus one avoids the prolongation of anaesthesia for a femoral vein injection, or the loss of rats from an experiment because of failures with tail-vein injections. It enables one to perform the assay with a mean control secretion of about 6 ml. from rats of about 200 g. body weight. Control secretion rates of the order of 2 ml./100 g. body weight/hr., such as are needed for the two-hour assay, are hard to obtain consistently even with rats of a good strain. In this work, as in the experiments of Visscher *et al.* (1954), some experiments have had to be excluded because the mean control secretion was less than 4 ml. This may cause serious waste of animals with some strains. Two features of the present method have helped to get over this difficulty. First, there is the use, already mentioned, of a three-hour collection period for the parenteral assay. Second, it was found that attention to the adequate nutrition and hydration of the animals during the fast from solid food greatly improves their performance. Furthermore, if rats with an average secretion of gastric juice are injected with 2.5 mg. cortisone acetate on the day of the experiment, the production of highly acid gastric juice is increased (unpublished observations); this phenomenon is being investigated and will be reported on in detail elsewhere.

Since complete vagotomy, when performed as an acute experiment, abolishes the interdigestive secretion in rats (Komarov, Shay, and Gruenstein, 1947; Harkens, 1947; Madden, Ramsburg, and Hundley, 1951), and reduces it by 80% to 90% in the chronically vagotomized preparation (Komarov *et al.*, 1947), it is clear that the secretion inhibited in this assay procedure is comparable with the night secretion of the duodenal ulcer patient. Vagotomy reduces the night secretion in these patients by 80% (Dragstedt, Woodward, Storer, Oberhelman, and Smith, 1950). This would seem to mark the procedure as one particularly relevant in the search for an agent which will be useful in controlling the hyperchlorhydria of these patients.

Ing (1946) showed that the methyl salts of the belladonna alkaloids, though intrinsically more potent than the corresponding tertiary compounds, are usually less potent locally as mydriatics. A similar impairment of activity in depressing gastric secretion in rats, apparently due to imperfect

absorption, was noted by Visscher *et al.* (1954) when hyoscine methyl bromide was given by the intestinal route.

The relative inactivity of the methyl derivative of atropine given as a mydriatic locally (as the methonitrate ; Ing, 1946) is in line with its relative inactivity by the intestinal route in depressing gastric secretion (Table II, col. iii). However, relative inactivity by the intestinal route does not seem to be a general feature of quaternary derivatives of atropine. The duodenal-intramuscular ED50 ratio of the propyl derivative is of the same order of magnitude as that obtained with atropine sulphate (Table II).

Summary

1. A method is described of studying the inhibition of gastric secretion in rats with the pylorus ligated.

2. Atropine methyl iodide, atropine ethyl bromide, atropine propyl iodide, atropine benzyl chloride, and atropine *p*-nitrobenzyl chloride were compared with atropine sulphate, by an enteral and a parenteral route on this preparation.

3. Parenterally, atropine methyl iodide is the most active derivative. Enterally, the sulphate is more active than any of the quaternary derivatives; of the latter, the methyl iodide is the most active.

4. The relative inactivity of the methyl derivative by the intestinal route is not a general characteristic of quaternary atropine derivatives.

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Brit. J. Pharmacol. (1956), **11**, 175.

SOME CENTRAL ACTIONS OF 5-HYDROXYTRYPTAMINE AND VARIOUS ANTAGONISTS

BY

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(RECEIVED JANUARY 5, 1956)

The pharmacological analysis of extracts of the central nervous system led to the conclusion that 5-hydroxytryptamine (HT) was present in such extracts and was distributed in a way that suggested it might play a part in the physiology of certain nervous centres and particularly those near the 3rd and 4th ventricles (Twarog and Page, 1953; Amin, Crawford, and Gaddum, 1954). A study of the distribution of the enzyme 5-hydrotryptophan decarboxylase, which forms HT, strengthened this conclusion (Gaddum and Giarman, 1956). The discovery that lysergic acid diethylamide (LSD) was a powerful and specific antagonist for HT (Gaddum, 1953; Gaddum and Hameed, 1954) suggested that the actions of this substance on the brain might be due to this antagonism (Gaddum, 1954; Woolley and Shaw, 1954). These actions were described by Stoll (1947) and have received much attention in recent years. The most obvious effects of small doses in man involve changes in personality and perception which have been compared with the symptoms of schizophrenia. The experiments described here were undertaken with the object of discovering whether a known central action of HT was in fact antagonized by LSD. Central actions of HT in cats, uncomplicated by general vascular effects, can only be observed by giving it intrathecally or into the cerebral ventricles. The effect of injecting HT into the lateral ventricle of a conscious cat had been described by Feldberg and Sherwood (1954). It was thought that the effects might be specifically antagonized by small doses of LSD.

METHODS

Metal cannulae were screwed into the skulls of cats anaesthetized with pentobarbitone, as described by Feldberg and Sherwood (1953), and injections were made through these cannulae into one lateral ventrical during the succeeding weeks or months. The effects of the injections were recorded merely by observing the behaviour of the cats.

The following drugs were used: 5-hydroxytryptamine creatinine sulphate (Abbott Laboratories), lysergic acid diethylamide tartrate (Sandoz, Ltd.), reserpine solution ("Serpasil," Ciba Laboratories), ergometrine maleate, 2-bromo-lysergic acid diethylamide tartrate ("Bol 148," Sandoz, Ltd.), 1 : 2-dimethyl 3-ethyl 5-dimethylamino-indole ("methylmedmain"), 5-benzyloxygramine, morphine HCl, methadone HCl, amphetamine sulphate, methylamphetamine HCl (Methedrine, Burroughs Wellcome & Co.).

Most drugs were dissolved in sterile 0.9% NaCl solution and injected in a volume not exceeding 0.2 ml. Methylmedmain was dissolved in the equivalent amount of 0.15N-HCl to make an approximately 3% solution of the base. Benzyloxygramine, the hydrochloride of which is not very soluble, was heated with the equivalent amount of dilute HCl and a 1% solution (in terms of the base) prepared by the addition of 0.9% NaCl solution. The mixture was slightly cloudy.

Doses of HT are given in terms of the base, of the other salts as weight of the salt.

RESULTS

HT.—The observations of Feldberg and Sherwood (1954) on the effects of HT on cats were in general confirmed. Doses of 0.1–0.6 mg. caused a curious lethargy during which the cats, which had been energetic and enterprising, became hesitant and retiring. They lost initiative and instead of wishing to run about they appeared anxious to return to their cages. There was diminished muscular tone, so that the body was apt to sag, but the cat could be roused. The respiration was very rapid during the first 30 min. after the injection.

When no other injections were made, these effects lasted at least 6 hr., but the cats were normal the next day. The effects observed by Feldberg and Sherwood apparently did not last so long as this. The reason for this difference in the duration of the effect is not known.

Reserpine.—The depressant actions of this drug on the central nervous system have been widely studied (Conferences, 1954, 1955). According to

Shore, Silver and Brodie (1955), and Pletscher, Silver and Brodie (1955), reserpine releases HT in the body, causing depletion of tissue HT and the appearance of a metabolite of HT in the urine. The depletion of HT in brain extracts has also been observed by Paasonen and Vogt (1956). It is therefore suggested that the effects of reserpine are due either to the disappearance of HT from the tissues, or to the flooding of the body with excessive amounts of this substance. The observation of Shore, Silver, and Brodie (1955) that reserpine and HT both increase the effects of hypnotics when injected intraperitoneally in mice is in favour of the second of these possibilities, and the results obtained with cats point to the same conclusion, since both drugs have a depressant action.

The intraventricular injection of 10 μg. reserpine had no obvious effect during 3½ hr. observation. In most of the experiments with this drug, however, it was given intraperitoneally in a dose of 0.25–0.5 mg./kg. in the afternoon or evening. This generally caused diarrhoea in the first few hours and the cats gradually became depressed; the next morning the sedation was marked, the pupils narrow, the nictitating membrane relaxed and the eyes nearly shut; the cat sat quietly in its cage and walked clumsily when disturbed. The inertia produced by reserpine was not unlike that due to HT, though the effects on the pupil and nictitating membranes were not observed with HT, and the ataxia was more pronounced with reserpine. It is possible that the mode of administration is responsible for some of these differences. Drugs given systemically are likely to reach sites inaccessible to drugs injected into the cerebral ventricles.

In one cat 0.3 mg. HT was injected intraventricularly at an interval of 16 hr. after reserpine. The only apparent result of this second injection was that the cat became even more lethargic.

LSD.—The injection of 1–20 μg. into the cerebral ventricle had no obvious effect. A dose of 1 μg./kg., given by the mouth to man, has profound subjective effects and it might therefore be expected that the injection of 20 μg. into the ventricle would have had a marked action on the cat. It is, of course, impossible to know what subjective effects may have been produced, but the cat's behaviour was apparently unaltered after this dose.

The injection of 0.2 mg. LSD intraventricularly caused minor symptoms such as salivation, and the injection of 0.8 mg. caused "sham rage." Cats which had been quiet and friendly became for a time unreasonable and intolerant. They hissed fiercely and bared their teeth when approached, and seemed to resent even the smallest movement or remark. These effects appeared in about 3 min. and lasted about 45 min.

The subcutaneous injection of 3 mg. LSD in one of these cats caused symptoms of the same kind lasting about 2 hr.

When 0.2 mg. HT and 0.8 mg. LSD were injected simultaneously, the effect of the LSD appeared first and after about 45 min. the cat appeared nearly normal. About 4 hr. after the injection it became lethargic, presumably owing to the continued action of the HT.

When 0.3 mg. HT was followed by 0.8 mg. of LSD 45 min. later the lethargy due to the HT was interrupted by the LSD and returned 3 hr. later. While the LSD was acting, the cat was as excited, intolerant and restless as if the drug had been given alone. During this phase its muscular strength appeared quite normal.

In an experiment with another cat, which was normally energetic, 0.6 mg. HT caused very marked apathy; the injection of 0.8 mg. LSD 45 min. later overcame the apathy and caused great excitement.

The effect of reserpine was also antagonized by LSD. A cat received reserpine (0.25 mg./kg. intraperitoneally) and 20 hr. later LSD (2 mg. subcutaneously). The reserpine had its usual depressant effect and the eyes were nearly shut, the pupils contracted, and the nictitating membrane relaxed. The LSD roused the cat in about 20 min. and made it restless and angry, but it was clumsy and did not move much. All the eye symptoms had disappeared—the eyes were open, the pupils dilated and the nictitating membranes retracted. This effect lasted about 1½ hr., after which the typical effects of reserpine returned—the cat became inert and the eye symptoms returned.

These experiments left no doubt that the apathy due to HT or reserpine temporarily disappeared under the action of LSD.

Ergometrine.—The molecule of ergometrine is very similar to that of LSD, but this drug is less active as an antagonist of the action of HT on rat's uterus (Gaddum and Hameed, 1954). Recent experiments (Blair, personal communication, 1955) indicate that its activity on the rat's uterus as an antagonist of HT is about 1/10 that of LSD.

Ergometrine (2–2.5 mg./kg. intravenously) is known to cause sham rage in cats (Brown and Dale, 1935).

When 2 mg. ergometrine maleate was injected intraventricularly the effect was similar to that of LSD, though not the same. After 10 min. the cat was salivating but sitting immobile. Its pupils then widened and after 25 min. it became ataxic. After about 40 min. it resented being approached, but

appeared frightened rather than angry. The cat was more ataxic and less active than it had been after LSD. This effect lasted for over 6 hr. The injection of 0.4 mg. had similar but smaller effects.

In another experiment, 0.3 mg. HT was followed by 1.5 mg. of ergometrine maleate 28 min. later, both drugs being injected through the cannula. The depressant effect of the HT was antagonized by the ergometrine. The cat which had become lethargic under the influence of HT became restless and apprehensive with dilated pupils about 20 min. after the second injection and remained in this condition for over 2 hr. It was somewhat ataxic and clumsy and not very active, but in other respects the effect of ergometrine was not widely different from that of LSD.

Brom LSD ((+)-2-bromo-lysergic acid diethylamide tartrate).—This substance acts like LSD as an antagonist of HT on rat's uterus, but does not act like LSD on the human brain. These facts provide evidence against the theory that the action of LSD on the human brain is due to this antagonism (Cerletti and Rothlin, 1955).

When a dose of 1 mg. of Brom LSD was injected into a cat's cerebral ventricle, it entirely failed to cause an exhibition of sham rage such as was regularly caused in the same cat by an equivalent dose of LSD (0.8 mg.).

In another experiment, 1 mg. of Brom LSD was injected 1 hr. after an injection of 0.3 mg. HT. No effect was seen except for salivation, but this cat had acquired the habit of salivating in response to all injections. An injection of 0.8 mg. LSD 1 hr. and 25 min. after the Brom LSD caused typical sham rage with dilatation of the pupils, hissing and considerable motor activity.

These results show that Brom LSD does not produce the sham rage and alertness seen after equivalent doses of LSD and that it does not prevent these effects being produced by a subsequent injection of LSD.

Methylmedmain (1 : 2 - dimethyl 3 - ethyl 5 - dimethylamino-indole).—This compound has been found to antagonize some of the peripheral actions of HT (Shaw and Woolley, 1954), and an attempt was therefore made to discover whether it also antagonizes the central action of HT in these cats. The dose was a large one. An isotonic solution of methylmedmain hydrochloride containing 6.2 mg. of the base was injected intraventricularly. Salivation and panting occurred in a few minutes followed, an hour after the injection, by inertia and crouching similar to that produced by HT itself. The action of this drug was quite different from that of LSD. It was clear that central antagonism to HT could not be demonstrated by this technique and no further injections were made.

5-*Benzyloxygramine*.—This was the most active of a series of new synthetic indole compounds studied by Gaddum, Hameed, Hathway, and Stephens (1954). The intraventricular dose was 2.1 mg. of the base dissolved with HCl in 0.2 ml. of saline; larger amounts could not be dissolved in a suitable volume of fluid. Salivation and a great acceleration of respiration began 10 min. after the injection and persisted for 2 hr. The cat was rather clumsy, but fairly alert and active. A dose of 0.3 mg. HT was then given and produced its usual effect. The injection of as much 5-benzyloxygramine as is practicable thus had no specific effect on the cat's behaviour and did not prevent the action of HT given later.

Morphine.—It is well known that morphine generally causes excitement in cats and depression in dogs. There is some evidence that it antagonizes the action of HT on peripheral tissues (Kosterlitz and Robinson, 1955).

A dose of 2 mg. was injected through the cannula. After 5 min. the cat salivated and defaecated. Its pupils dilated; it ran round the room and appeared agitated and angry. The effect lasted about 4 hr. and had nearly disappeared after 6 hr.

On another day 0.3 mg. HT was injected and the normal depressant effect observed for 44 min. Morphine HCl (1 mg.) was then injected through the cannula and the cat was immediately aroused. After 30 sec. it jumped into the cage with its ears cocked. Within 10 min. it was excited, as it had been after morphine alone. In another cat the effect of HT was reversed in much the same way by the subcutaneous injection of 3 mg. morphine.

In the experiment described above, when reserpine had been injected intraperitoneally and HT through the cannula, an injection of morphine (1 mg.) was given, also through the cannula, 50 min. after the injection of HT. The effect of this injection came on more slowly than those of previous injections of morphine, but after 17 min. it was apparent that the morphine had antagonized most of the effects of both the other drugs. The pupils had partially dilated and the eyes were half open, only the nictitating membranes remained relaxed. The cat now took an increased interest in its surroundings and resisted when picked up. After 45–60 min. the effects of morphine were fully developed and the cat salivated and was excited and unmanageable.

These experiments showed that morphine had an action not unlike that of LSD.

Methadone.—The central effects of this synthetic analgesic are similar to those of morphine. It causes excitement in cats. The injection of 10 mg. methadone subcutaneously caused restless excitement with dilated pupils increasing gradually during the first 1 hr. and lasting about 3 hr. When this same dose was injected at a time when the cat was lethargic following the injection of 0.3 mg. HT intraventricularly, the lethargy disappeared and the cat became excited in much the same way as without HT.

In another cat reserpine (0.25 mg./kg.) was injected intraperitoneally. The next morning, 20 hr. after the injection, this cat was inert with its eyes nearly shut, its pupils contracted and its nictitating membranes relaxed. Methadone (10.5 mg.) was then injected subcutaneously. About an hour later this cat roused itself and rushed about clumsily. The pupils were now widely dilated, but the nictitating membranes were still relaxed.

Amphetamine is known as a central stimulant. It inhibits amine oxidase *in vitro* and it is thought that some of its actions *in vivo* may be due to this effect. The effect of the intraventricular injection of this drug was unexpected. When 0.5 mg. was injected by this route the cat became lethargic for 3–4 hr., after first vomiting. The rate of respiration rose from 37 to 200 during the first 20 min. and then fell to 47 during the next 30 min.

In another experiment, 0.3 mg. HT was injected intraventricularly and followed by 0.1 mg. amphetamine sulphate 30 min. later by the same route. As this second injection did not seem to have affected the normal response to HT, it was followed by the injection of 0.5 mg. amphetamine sulphate 84 min. later. The cat now vomited and urinated and then became more inert than ever. The amphetamine seemed to have intensified the normal depressant action of the HT.

An experiment was then performed in which 0.3 mg. HT was injected into the ventricle and amphetamine sulphate (15 mg./kg.) given subcutaneously 75 min. later. Pupillary dilatation was obvious in a few minutes, and during the course of the next hour the cat became alert, moving its head around a great deal as if searching for something, and running away when approached. Its muscular tone was improved though, except for the incessant head movement, it kept in one place unless stirred. The alertness and searching movements contrasted sharply with the inertia seen after HT alone or after HT and intraventricular amphetamine. The cat remained in the same state for several hours, becoming gradually fiercer and hissing when approached. The effect of amphetamine given subcutaneously to normal cats follows the same pattern and time course. These results show clearly that, in contrast to morphine and LSD which act similarly when given by either route, amphetamine acts differently when injected into the cerebral ventricles and subcutaneously.

Methylamphetamine (Methedrine) and LSD.—The actions of methylamphetamine resemble those of amphetamine.

A few observations were made of the actions of LSD and methylamphetamine in man. One of us took, on four occasions, 30–86 μg. LSD by the mouth and experienced some of the known effects of this drug, such as a feeling of irresponsibility and euphoria, increased awareness of shapes and colours, shimmering of peripheral vision, and a dreamy feeling that sensations did not represent real objects. These subjective effects were accompanied by apparent intoxication and a loss of the power of concentration. Groups of 8 digits were read out from a table of random numbers and the subject tried to repeat them immediately. The normal average score in this test was nearly 100%, but under the action of LSD the score fell to a low figure and fatigue developed rapidly. There was no obvious incoordination, but some loss of the power of reproducing a simple drawing. These effects reached a maximum in about 1½ hr. and then gradually disappeared during the next 3–4 hr.

It was thought that if these effects were due to the inhibition of physiological HT they might be overcome by the administration of methylamphetamine. This drug might be expected to increase the concentration of HT in the brain by inhibiting amine oxidase, and the increased amount of HT might overcome the effect of LSD. This line of reasoning involved various untested hypotheses, but at least it had the virtue of suggesting an experiment which could easily be carried out.

After 3 weeks without drugs 10 mg. methylamphetamine hydrochloride was swallowed at the same time as 86 μg. of LSD. In this experiment there were no obvious signs of intoxication and the ability to repeat random numbers was not impaired. On the other hand, LSD produced the same subjective sensations in this experiment as it did in the other experiments. The conclusion was reached that methylamphetamine had inhibited the objective signs of intoxication by LSD, but had not altered its subjective effects. Self-control was improved, but this is a known effect of methylamphetamine and there is no special reason to believe that it depends on the interaction of methylamphetamine and HT.

Discussion

The first object of these experiments was to demonstrate antagonism between the central effects of HT and LSD, and this was done. The effect of HT was antagonized by LSD, but there was no evidence that the effect of LSD was antagonized by HT. When LSD was given first its effect was so brief that there was no time for the HT to act. When HT was given either before the LSD or at the same time, it caused no clear change in the response to LSD. These results raise doubts about the theory that the central stimulant action of LSD is due to interference with natural mechanisms mediated by HT, and these doubts are strengthened by the results obtained with other drugs.

Experiments with morphine, methadone, ergometrine and subcutaneous amphetamine showed that, although the effects of these drugs differed in detail, they all had the effect of rousing cats from lethargy due to HT. Morphine and methadone also antagonized the main effects of reserpine. All these drugs have in common that they cause sympathetic discharge and the antagonism they exert may come in the category of "arousal" by sympathetic stimulation (Bonvallet, Dell, and Hiebel, 1954).

Brom LSD, methylmedmain and 5-benzyloxygramine all antagonize the effect of HT on the rat's uterus, but they did not rouse cats and there was no evidence that they prevented the central action of HT injected into the ventricle.

These results suggest that the antagonisms studied in these experiments are unspecific and have no relation to the antagonism between HT and LSD in their effects on peripheral organs. The only actions observed, however, were the gross effects of large doses, and the results do not exclude the possibility that some of the central effects of small doses of LSD are due to interference with natural mechanisms mediated by HT. The results of the experiments on man suggest that the central actions of LSD are complex and that antagonists may act on some effects and not on others.

Shore, Silver, and Brodie (1955) observed a central antagonism of LSD to the potentiating action of reserpine on the sleeping time of anaesthetized mice. The dose of LSD (10 mg./kg.) was even greater than that used in the present experiments on cats, and the possibility of a non-specific antagonism might also have to be considered in this work.

It must be remembered that there is no evidence that the effect of intraventricular HT is exerted directly on neurones and not mediated through a constriction of cerebral blood vessels in regions near the ventricles. The depression produced by injecting large doses of adrenaline into the ventricles (Feldberg and Sherwood, 1954) bears much resemblance to the effect of intraventricular HT, and vascular effects may play some part at least in the responses to both drugs when they are administered by this route.

Summary

1. The depressant action of HT on the brains of cats is antagonized by LSD, ergometrine, morphine, methadone and amphetamine, but not by 2-bromo-LSD, 5-benzyloxygramine or methylmedmain. The sedation produced by reserpine is antagonized by LSD, morphine and methadone.

2. These antagonisms are probably not related to the specific antagonism between HT and LSD on peripheral tissues.

3. Methylamphetamine inhibits some but not all the central actions of LSD in man.

We are grateful to the following for gifts of drugs: to Abbott Laboratories for 5-hydroxytryptamine; to Sandoz, Ltd., for "LSD 25" and "Bol 148"; to Ciba Laboratories for Serpasil; to Glaxo Laboratories for benzyloxygramine; and to Dr. D. W. Woolley for methylmedmain.

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EFFECT OF CHLORPROMAZINE ON ADRENALINE VASOCONSTRICTION IN MAN

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(RECEIVED JANUARY 10, 1956)

Chlorpromazine is a synthetic alkyl amino derivative of phenothiazine, with a chemical structure very similar to that of promethazine. It has, however, less antihistaminic activity and is a more powerful central depressant. Antagonism to adrenaline has been described in animals (Courvoisier, Fournel, Ducrot, Kolsky, and Koetschet, 1953), and in man (Foster, O'Mullane, Gaskell, and Churchill-Davidson, 1954), but the nature of this action has not been fully elucidated.

The present study is a quantitative assessment of the antagonism between chlorpromazine and adrenaline in the blood vessels of the hands of healthy human subjects.

METHODS

Twenty-two tests were performed on nine male medical students and colleagues aged from 21 to 50 years. Each subject lay at rest on a comfortable couch in the laboratory for at least half an hour before the start of the test. The blood flow in both hands was measured regularly at half minute intervals by venous occlusion plethysmography under standard conditions: the laboratory temperature was maintained at 22–24° C., and the metal plethysmographs were filled with water (Barcroft and Edholm, 1943) kept at 32–34° C. (Duff, 1952). Blood flow was calculated and expressed as ml./100 ml. hand volume/min.

After anaesthetizing the overlying skin, a unilateral intra-arterial infusion of saline (0.9% w/v NaCl soln.) was given through a needle inserted into the brachial artery on one side just above its bifurcation at the elbow. Saline was delivered at the rate of 3 ml./min. by an electric infusion machine from one or other of two 50 ml. syringes, connected to the intra-arterial needle by a polythene tube and adaptor. Intravenous infusions were similarly given into a superficial vein of the opposite arm, using a second machine. Solutions of adrenaline (synthetic (−)-adrenaline tartrate, B.D.H.) and chlorpromazine ("Largactil," M. & B.) were prepared by serial dilution with saline. The potency of the adrenaline was maintained by adding 0.1 mg. ascorbic acid to each pint of saline used in the infusions (Gaddum, Peart, and Vogt, 1949).

The effect of chlorpromazine on the peripheral constriction caused by adrenaline was studied in two series of experiments. In the first of these the effect of *intra-arterial* chlorpromazine on the vasoconstriction produced by intra-arterial and by intravenous adrenaline was measured; this study also enabled the direct action of chlorpromazine alone on the calibre of the vessels to be assessed. In the second series of experiments, the effect of *intravenous* chlorpromazine on the constriction produced by intra-arterial adrenaline was studied.

Action of Intra-arterial Chlorpromazine on the Effects of Intra-arterial and Intravenous Adrenaline

The course of a typical experiment is illustrated in Fig. 1. After a control period of approximately 10 min. during which saline alone was given, three 4-min. infusions of adrenaline were alternated with control saline infusions for at least 4 min. Two infusions of adrenaline in a concentration of 0.5 μg./min. were given intra-arterially and one infusion of adrenaline (10 or 15 μg./min.) was given intravenously. Chlorpromazine was then infused into the artery in a total dose of 1.2 mg. at the rate of 200 μg./min. for 6 min. This amount was calculated to be the approximate fraction of a 50 mg. i.v. dose which would be expected to reach the brachial artery at the elbow (Barcroft and Swan, 1953). The intra-arterial and intravenous infusions of adrenaline were then repeated in the same concentration and order as before the chlorpromazine.

The blood flow in both hands normally fluctuates from moment to moment, as the result of systemic influences—especially variations in sympathetic activity —affecting both hands about equally (Cooper, Cross, Greenfield, Hamilton, and Scarborough, 1949; Duff, 1952). These phasic variations are not influenced by intra-arterial or intravenous infusions of saline alone, but allowance must be made for them before changes in hand blood flow can be attributed solely to the action of substances infused into the brachial artery. Thus, in the experiment depicted in Fig. 1, the mean flows, in the control period before the first intra-arterial infusion of adrenaline, averaged (*A*) 15.1 ml./100 ml. hand volume/min. in the test right hand and (*a*) 17.1 ml. in the control left hand. During the infusion of 0.5 μg./min.

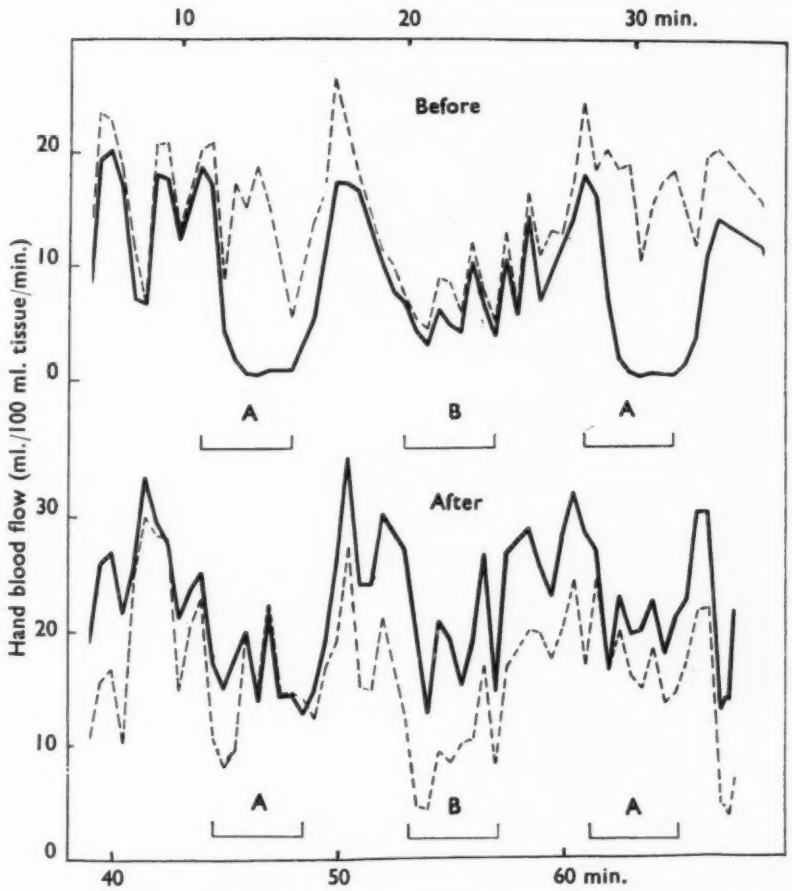

FIG. 1.—Response of hand blood flow in one subject (O. R., ♂) to adrenaline, intra-arterially (A, 0·5 μg./min. for 4 min.) and intravenously (B, 10 μg./min.) before (upper records) and after (lower records) chlorpromazine (1·2 mg. in 6 min., starting at 37 min. on the record) infused into right brachial artery. Solid lines, right hand; broken lines left hand.

adrenaline, the corresponding values were (*B*) 1.3 ml. and (*b*) 12.6 ml. for the right and left hands respectively.

Since, normally, *A*/*a* approximately equals *B*/*b*, an estimate of *B* is given by the expression *Ab*/*a*, assuming adrenaline to have had no effect on the blood flow in the test hand. This estimate is denoted by the symbol $E \simeq Ab/a$ and averaged 11.1 ml. in this instance. The measured value for *B*, however, was 1.3 ml. The difference between these two values—that is, between the estimated or expected value *E*, derived from *Ab*/*a*, and the measured value *B*—represents the net effect of adrenaline on the flow in the test right hand. Thus, $B-E=1.3-11.1=-9.8$, and indicates a net reduction in flow of nearly 10 ml. during the infusion of adrenaline. When this is expressed as a percentage, $[B-E/E]\%$, the infusion of 0.5 μg./min. adrenaline can be said to have caused a decrease in mean flow of $\frac{9.8\times100}{11.1}$ or 88% of the expected value. Similarly, with the second intra-arterial infusion of adrenaline (Fig. 1), the mean decrease in flow was 84% of the expected value. This method of analysis (Duff, 1952) was applied to the results of *all* intra-arterial infusions.

With intravenous infusions, any drug effect involves both hands. Hence the change in flow in either hand, expressed as a percentage of the previous level of flow in that hand, provides an estimate of the peripheral constrictor or dilator effects of drugs given intravenously.

Thus, in the experiment depicted in Fig. 1, the effect of the first intravenous infusion of adrenaline (10 μg./min.) on the flow in the right test hand, as given by the expression $[B-A/A]\% = [5.5-12.7/12.7]\%$. Similarly, the effect of this infusion on the flow in the left hand is obtained from $[b-a/a]\% = [6.2-15.9/15.9]\%$. The mean percentage reduction in flow with this intravenous infusion of adrenaline was therefore 57% in the right hand and 61% in the left hand.

Action of Intravenous Chlorpromazine on the Effects of Intra-arterial Adrenaline

In these experiments, an infusion of adrenaline (0.5 μg./min. for 4 min.) was given before and after an intravenous infusion of chlorpromazine (50 mg. in 10 min.).

In view of the possible dangers attending systemic administration of both substances together, subjects receiving intravenous chlorpromazine were not given intravenous adrenaline.

RESULTS

Action of Intra-arterial Chlorpromazine on the Effects of Intra-arterial Adrenaline

The results of thirteen intra-arterial infusions of adrenaline (0.5 μg./min.) before and after the infusion of 1.2 mg. chlorpromazine into the brachial artery are summarized in Table I. In each test, *A* and *a* are the *mean* values of six flows recorded simultaneously in test and control hands respectively *before* the infusion of adrenaline: *B* and *b* are the *mean* values of six flows similarly recorded in these hands *during* the adrenaline infusion.

Before chlorpromazine, intra-arterial adrenaline caused a decrease in flow in the test hands which averaged 75% (Table I, col. *B*–*E*/*E*). After 1.2 mg. chlorpromazine had been infused into the brachial artery, the level of flow in the test hand increased;

TABLE I

RESPONSE TO INTRA-ARTERIAL ADRENALINE (0·5 μG./MIN. FOR 4 MIN.) BEFORE AND AFTER INFUSING CHLORPROMAZINE (1·2 MG. IN 6 MIN.) INTO THE BRACHIAL ARTERY

A, *a* = means of 6 measurements of hand blood flow during 3 min. before adrenaline in test and control hands, respectively; *B*, *b* = corresponding means during first 3 min. of adrenaline period; *E* = *Ab*/*a*. Mean blood flows in cols. *A*, *B*, *a*, *b*, in ml./100 ml. tissue/min.

Expt. No.	Before Chlorpromazine					After Chlorpromazine				
	Test Hand		Control Hand			Test Hand		Control Hand		
	A	*B*	*a*	*b*	$\frac{B-E}{E}$	*A*	*B*	*a*	*b*	$\frac{B-E}{E}$
1	10·9	4·9	5·9	5·1	−48	18·0	9·7	10·2	6·5	−16
2	9·2	1·4	3·9	4·9	−88	19·5	16·4	12·7	16·4	−35
3	15·1	1·3	17·1	12·6	−88	20·1	16·3	14·5	16·8	−30
4	12·9	2·2	16·1	16·9	−84	24·1	19·9	20·5	16·5	−02
5	7·3	2·0	5·9	6·9	−76	20·9	14·7	8·7	8·5	−28
6	8·9	2·2	9·8	9·5	−74	17·6	10·2	9·6	9·1	−39
7	16·4	2·7	12·7	13·9	−85	26·9	20·9	16·6	14·1	−08
8	8·9	2·2	9·8	9·5	−74	14·8	7·7	7·5	7·4	−47
9	17·2	3·9	16·7	11·7	−68	29·0	19·1	25·0	21·9	−25
10	3·4	0·9	3·5	3·1	−70	9·8	5·3	2·3	2·0	−38
11	22·7	1·2	17·8	12·9	−93	38·9	15·4	17·9	16·4	−57
12	3·4	0·9	3·5	3·1	−70	10·7	7·3	1·9	1·4	−08
13	12·1	3·3	8·7	6·1	−61	13·0	13·7	9·3	12·8	−23
Mean	11·4		10·1		−75	20·3		12·1		−27

adrenaline then caused a reduction in flow averaging only 27%. This change in mean percentage reduction with adrenaline, from 75% before to 27% after chlorpromazine, is considerable (Table I). The effect of adrenaline on the hand blood flow, before and after chlorpromazine, in each of the thirteen tests, is shown diagrammatically in Fig. 2.

The *net* change in flow during the infusion of adrenaline can be calculated from Table I, using the formula *B*–*E*. The mean change in flow with intra-arterial adrenaline was 7.9 ml. before and 5.7 ml. after chlorpromazine: the difference between these means is not significant (S.E. = 1.33, t = 1.7). Adrenaline therefore caused much the same *net* reduction in flow before and after the drug infusion.

The blood flow in the hand which had been infused with chlorpromazine always increased (Table I, col. *A*). The increase started before the end of the drug infusion, and the raised level of flow was still present at the end of the experiment. The amount of vasodilatation varied in different subjects; the average increase in flow in the hand which had received chlorpromazine was from a mean of 11.4 ml. to a mean of 20.3 ml. After accounting for any bilateral changes in flow, this represents a mean rise in flow of 49% due to chlorpromazine alone.

The flow in the opposite (non-infused) hand rose in ten experiments and fell in three during the infusion of chlorpromazine (Table I, col. *a*): the mean change in flow, however, was only 3.1 ml. It may therefore be assumed that the amount of chlorpromazine reaching the general circulation was not sufficient to influence the flow appreciably in the non-infused hand.

The mean changes in flow in both hands during these infusions are shown in Fig. 3.

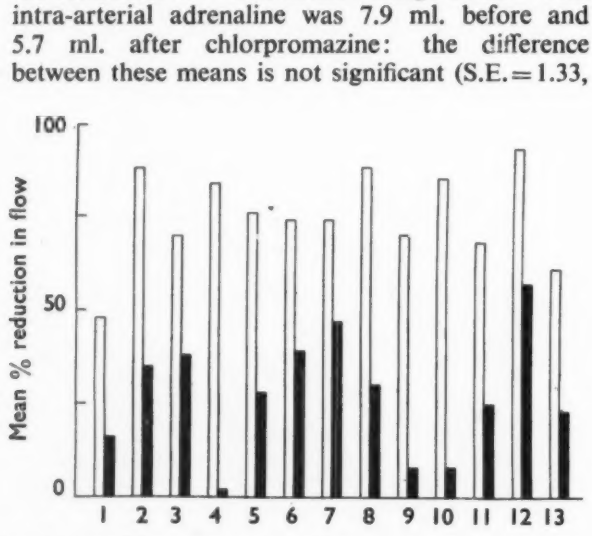

FIG. 2.—Blood-flow responses, in 13 different hands, to intra-arterial adrenaline (0·5 μg./min. for 4 min.) before (open rectangles) and after (solid rectangles) intra-arterial chlorpromazine (1·2 mg. in 6 min.).

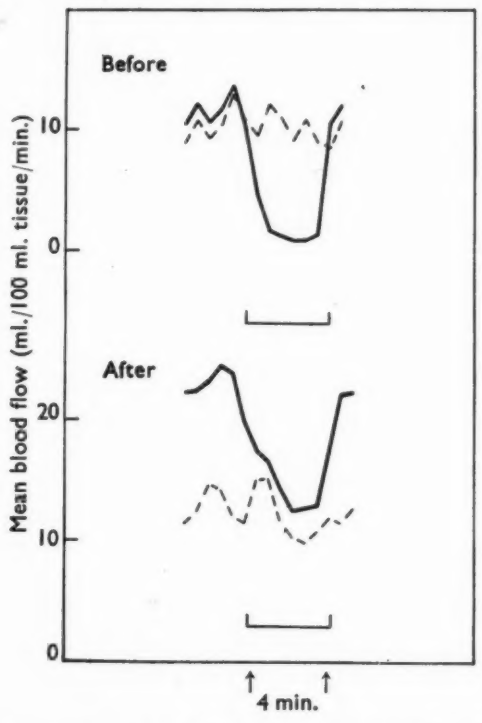

FIG. 3.—Mean blood flow in test hands (solid lines) and control hands (broken lines) during infusions of intra-arterial adrenaline (0·5 μg./min. for 4 min.) before (upper records) and after (lower records) intra-arterial chlorpromazine (1·2 mg. in 6 min.).

TABLE II

RESPONSE TO INTRAVENOUS ADRENALINE (10 TO 15 μG./MIN.) BEFORE AND AFTER INFUSING CHLORPROMAZINE (1·2 MG. IN 6 MIN.) INTO THE BRACHIAL ARTERY

A, *B*, *a*, *b*, as in Table I. Blood flows in ml./100 ml. tissue/min.

Expt. No.	Before Chlorpromazine						After Chlorpromazine					
	Test Hand		Control Hand				Test Hand		Control Hand			
	A	*B*	*a*	*b*	$\frac{B-A}{A}$%	$\frac{b-a}{a}$%	*A*	*B*	*a*	*b*	$\frac{B-A}{A}$%	$\frac{b-a}{a}$%
1	10·3	4·9	6·6	2·5	−52	−65	20·1	10·2	9·4	3·1	−49	−67
2	12·7	5·5	15·9	6·2	−57	−61	27·5	16·9	18·3	7·8	−39	−57
3	15·0	6·2	10·2	4·4	−59	−57	35·9	19·8	16·3	5·9	−45	−65
4	4·9	1·9	4·8	2·5	−61	−46	21·3	7·7	8·7	3·4	−64	−61
5	16·7	4·6	10·6	3·9	−72	−63	30·2	13·9	20·3	6·9	−54	−66
6	3·1	1·5	2·6	1·3	−52	−50	14·1	7·8	2·5	1·1	−45	−56
Mean					−59	−57					−49	−62

Action of Intra-arterial Chlorpromazine on the Effects of Intravenous Adrenaline

Adrenaline was given intravenously to six subjects before and after an intra-arterial infusion of 1.2 mg. chlorpromazine. The characteristic effects of systemic adrenaline, such as tachycardia and pallor, occurred in all subjects, three of whom received 10 μg./min. and three 15 μg./min. The blood flow in the hands (Fig. 1 and Table II, cols. *B–A*/*A* and *b–a*/*a*) decreased on average by 59% on one, and by 57% on the other, side.

When intravenous adrenaline was given after infusing chlorpromazine into the brachial artery, the reduction in flow averaged 49% in the test hand (Table II, col. *B–A*/*A*). The difference between the reduction of 59% before and 49% after chlorpromazine, though small, is significant (S.E.=3.0, t=2.8). By contrast, the opposite hand, which had not received chlorpromazine, responded to the second intravenous infusion of adrenaline with a reduction in flow of 62% (Table II, col. *b–a*/*a*). This value is not significantly different from the previous mean of 57% (S.E.=2.8, t=1.5).

The effect of intravenous adrenaline was therefore slightly but significantly reduced in the hand that had been infused with 1.2 mg. chlorpromazine.

Action of Intravenous Chlorpromazine on the Effects of Intra-arterial Adrenaline

The effect of intravenous chlorpromazine on the response to intra-arterial adrenaline was tested in three subjects (Table III). In the control period before chlorpromazine, the reduction in flow (Table III, col. *B–E*/*E*) with intra-arterial adrenaline averaged 75% in the test hand. The mean percentage reduction was only 22% after intravenous chlorpromazine, when the level of flow had increased by at least 300%.

The *net* reduction in flow with adrenaline, *B–E*, was not, however, affected by chlorpromazine; the mean value was 4.5 ml. both before and after the chlorpromazine infusion.

The subjects were given 50 mg. chlorpromazine intravenously over 10 min. and rapidly became apathetic and drowsy. A rise in flow in both hands was apparent within 2 min. of the start of the infusion: the highest level of flow was recorded after about 6 min., when only half the total dose had been given. The rise varied between 300 and 400% in each hand (Table III, cols. *A* and *a*) and persisted until the end of the experiment.

TABLE III

RESPONSE TO INTRA-ARTERIAL ADRENALINE (0·5 μG./MIN. FOR 4 MIN.) BEFORE AND AFTER INFUSING CHLORPROMAZINE (50 MG. IN 10 MIN.) INTRAVENOUSLY

Col. headings as in Table I. Blood flows in ml./100 ml. tissue/min.

Expt. No.	Before Chlorpromazine					After Chlorpromazine				
	Test Hand		Control Hand			Test Hand		Control Hand		
	A	*B*	*a*	*b*	$\frac{B-E}{E}$%	*A*	*B*	*a*	*b*	$\frac{B-E}{E}$%
1	4·1	1·3	4·0	3·5	−64	19·9	14·8	22·0	19·3	−15
2	5·4	1·2	3·4	2·9	−74	27·8	20·6	27·6	25·1	−19
3	7·9	1·3	5·7	6·5	−86	21·7	15·4	20·9	20·7	−28
Mean					75					22

Discussion

Peripheral vasodilatation after chlorpromazine has been clearly demonstrated. Intra-arterial infusions of 1.2 mg. produced a mean rise in flow in the infused hand of 49%. This is the increase attributable to the drug alone, for the analysis (Methods and Table I) has taken account of any bilateral changes in flow. A similar increase in hand blood flow was found by Foster *et al.* (1954): intra-arterial infusions of 0.06 to 0.8 mg. chlorpromazine caused an average increase in flow in the infused hand of 50%. There is therefore good evidence that the drug has a direct action on the peripheral vessels.

When chloromazine was infused intravenously, in a dose (50 mg.) calculated to give approximately the same local concentration in the hand as in the intra-arterial experiments, the increase in flow was much greater, and averaged three or four hundred per cent in either hand. Intravenous chlorpromazine, therefore, produced a rise in hand blood flow almost eight times that found after comparable doses given intra-arterially. A similar disparity between the degree of vasodilatation after comparable intravenous and intra-arterial infusions was noted by Foster *et al.* (1954). Duff, McIntyre, and Butler (1956) found vasodilatation of a similar order. Peripheral vasodilatation after intravenous chlorpromazine would therefore appear to result from both local and systemic actions. Laborit and Huguenard (1951) suggested that many of the cardiovascular effects of chlorpromazine could be explained by a central inhibition of autonomic activity. Holtzbauer and Vogt (1954) have shown, however, that one form at least of central sympathetic activity is not inhibited by chlorpromazine—the stimulant action of morphine on hypothalamic sympathetic centres in cats. In the small group of subjects given intravenous chlorpromazine in this investigation, it was noted that the stimulant effect of a distended bladder caused subjective effects and a temporary decrease in hand blood flow. Moreover, all three subjects, though drowsy and disinclined to move, were able to stand and compensate for any postural hypotension within half to three-quarters of an hour of the end of the infusion. Chlorpromazine does not, therefore, cause complete inhibition of central vasomotor activity. Peripheral vasodilatation after intravenous chlorpromazine may result in part from other actions of the drug and may possibly involve changes in metabolism. Whatever the mechanism, the striking changes in peripheral flow should not be ignored when chlorpromazine is given in clinical practice.

Antagonism between chlorpromazine and adrenaline was first demonstrated by Courvoisier *et al.* (1953): the drug was found to abolish, and even reverse, the pressor action of adrenaline, and to afford some protection against its toxic effects. Hence chlorpromazine was described as a specific adrenaline antagonist, therein resembling dibenamine (Huidobro, 1954). Reduction of peripheral adrenaline vasoconstriction has been demonstrated in man (Foster *et al.*, 1954), but evidence of reversal was not conclusive (Foster *et al.*, 1954). The present investigation showed that the mean reduction in flow with intra-arterial adrenaline (Table I) was 75% before and 27% after intra-arterial chlorpromazine: chlorpromazine reduced the effect of adrenaline by about one half. These figures, however, represent responses to adrenaline expressed as a percentage of the control level of flow, after accounting for bilateral changes (see Methods). After chlorpromazine this control level was considerably greater. Calculation of the net alteration in mean flow (*B–E*), which simply measures the volumetric *reduction* in flow, showed no significant difference in the *net* effect of adrenaline after chlorpromazine. Thus adrenaline still caused much the same total reduction in flow, in the hand into which chlorpromazine had been infused; but since the initial level of flow was higher as the result of chlorpromazine the proportionate reduction was correspondingly less. The difficulty of deciding which is the more accurate measure of drug activity —the absolute or the relative change in response— arises in any biological assay. Since there was no change in the net response to adrenaline it might be

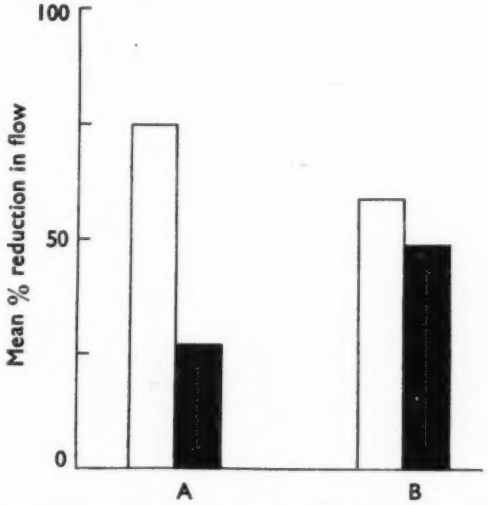

FIG. 4.—Mean response in all tests to adrenaline, intra-arterially (A, 0·5 μg./min. for 4 min.), and intravenously (B, 10 μg./min. for 4 min.), before (open rectangles) and after (solid rectangles) chlorpromazine intra-arterially (1·2 mg. in 6 min.).

argued that there is no antagonism, at least peripherally, between adrenaline and chlorpromazine in man. It may also be contended that much of the antagonism demonstrated in animals is attributable to peripheral vasodilatation, and not to a specific adrenaline antagonism. The increase in peripheral blood flow may therefore be an important factor in the protection afforded by chlorpromazine against adrenaline shock in animals.

It was interesting that chlorpromazine was much more effective against intra-arterial than against intravenous adrenaline (Tables I and III, Fig. 4). Reactivity to adrenaline may have been increased after a single intravenous dose (Cannon and Rosenblueth, 1949). The *net* change in flow was indeed greater by about 50% in both hands after the second intravenous infusion of adrenaline, but the *percentage* reduction in the hand which had *not* received chlorpromazine was not significantly increased. These results emphasize the complex nature of drug antagonism.

Summary

1. The effects of intra-arterial and of intravenous infusions of chlorpromazine on the blood vessels of the hands of healthy men have been assessed by venous occlusion plethysmography.

2. The volume of blood flowing through the hand increased by an average of about 50% after intra-arterial (1.2 mg.) and by about 400% after intravenous (50 mg.) chlorpromazine.

3. The percentage reduction in flow with intra-arterial (0.5 μg./min.) adrenaline averaged 75% before and 27% after the infusion of 1.2 mg. chlorpromazine into the brachial artery; but the *net* reduction in flow with intra-arterial adrenaline was not significantly different after chlorpromazine.

4. The average percentage reduction in flow in the hand with intravenous adrenaline was slightly but significantly decreased by the infusion of 1.2 mg. chlorpromazine into the brachial artery.

5. No reversal of peripheral adrenaline vasoconstriction was found even after intravenous chlorpromazine.

6. The results are discussed in relation to the problem of antagonism between adrenaline and chlorpromazine.

The authors have pleasure in expressing their indebtedness to Professor H. Barcroft for constant encouragement, advice and valuable criticism; to colleagues and medical students who volunteered as subjects; and to Mr. G. T. Hales for efficient technical assistance. Certain expenses were defrayed by a grant from the Endowment Fund of St. Thomas's Hospital. Chlorpromazine was supplied by May & Baker, Ltd.

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IDENTIFICATION OF 5-HYDROXYTRYPTAMINE IN THE STING OF THE NETTLE *(URTICA DIOICA)*

BY

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(RECEIVED JANUARY 26, 1956)

Emmelin and Feldberg (1947) showed that there were three smooth-muscle stimulating substances in the sting of the nettle: acetylcholine (ACh), histamine, and a third they did not identify. They described six properties of this unidentified third substance: constriction of the blood vessels of rabbit's ear; stimulation of *in vitro* preparations of rabbit's and guinea-pig's small intestine; lowering of rabbit's blood pressure; solubility in ether; and instability to boiling in alkaline solution. Since these properties coincided with those of 5-hydroxytryptamine (5-HT) subsequently described, we explored the possibility that the third substance of Emmelin and Feldberg might be 5-HT. Demonstration that this was so has already been briefly reported (Chesher and Collier, 1955) and the present paper sets out the detailed evidence on which identification rests and describes further investigations of nettle sting.

MATERIALS AND METHODS

For ease of supply we used the dioecious nettle, *Urtica dioica*. Both sexes showed the characteristic activity; but in all except a very few experiments female plants were used. Twenty-five or more individual stings were removed with forceps from the stalk or leaf and suspended in 0.5 ml. of the Ringer solution appropriate to the preparation in use. Acetylcholine chloride, atropine sulphate, 5-hydroxytryptamine (serotonin) creatinine sulphate, lysergic acid diethylamide tartrate (LSD), mepyramine maleate, and tryptamine hydrochloride were used and weights given are those of the salts. Dried whole wasp venom apparatus was used for reference as a source of kinin (Jaques and Schachter, 1954).

Preparations of rat's colon were suspended in a 25 ml. bath at 22–24° C. in an oxygenated Ringer solution containing: NaCl, 0.9%; KCl, 0.04%; $CaCl_2$, 0.003%; $NaHCO_3$, 0.015%; glucose, 0.1%, as described by Gaddum, Peart, and Vogt (1949). For rat's uterus the temperature was adjusted to 29° C. and the concentration of calcium in the Ringer solution doubled. For guinea-pig's ileum a temperature of 35–37° C. was used and the Ringer adjusted to contain 0.024% $CaCl_2$, 0.05% $NaHCO_3$ and 0.0005% $MgCl_2$ (Feldberg, 1951). To antagonize ACh on all these preparations, 24 ng./ml. atropine, and to antagonize histamine on the guinea-pig's ileum, 80 ng./ml. mepyramine, were added to the Ringer solution. For most preparations of the uterus, young rats received on the previous day 20 μg. stilboestrol in oil by the intramuscular route. This pretreatment was omitted before experiments on a possible fourth substance in nettle sting.

In preparing extracts for chromatography, leaf and stalk of nettle were extracted with acetone in a chill room at 4° C. and the extract concentrated by evaporation at this temperature for 3 days. Unidirectional descending paper chromatograms were run on Whatman No. 1 paper at 4° C. for 18 hr., using as solvent *n*-butanol-acetic acid (10 to 1) saturated with water. 1% *p*-Dimethylaminobenzaldehyde (Ehrlich's reagent) in aqueous *n*-butanol containing 0.5% HCl was used as developer.

RESULTS

Comparison of Third Substance with 5-HT.—When nettle sting suspensions were applied to the rat's uterus in the presence of atropine, they evoked a contraction which could be attributed to third substance. Since LSD antagonized this contraction, we compared its antagonism of third substance and of 5-HT. In a typical experiment (Fig. 1) a dose of LSD reduced the response to 5-HT by 51% and the response to third substance by 50%. This antagonism was specific, since, in the absence of atropine, LSD did not reduce the response of the preparation to ACh.

Large doses of tryptamine or 5-HT desensitize the guinea-pig's ileum towards themselves, but not to drugs of other types (Gaddum, 1953). Large doses of tryptamine also desensitized the guinea-pig's ileum towards nettle stings in the presence of atropine and mepyramine. For example, at the beginning of one experiment, 30 nettle stings provoked a contraction of the same height as 0.3 μg. 5-HT. The preparation was then exposed for 15 min. to 300 μg. tryptamine and the bath

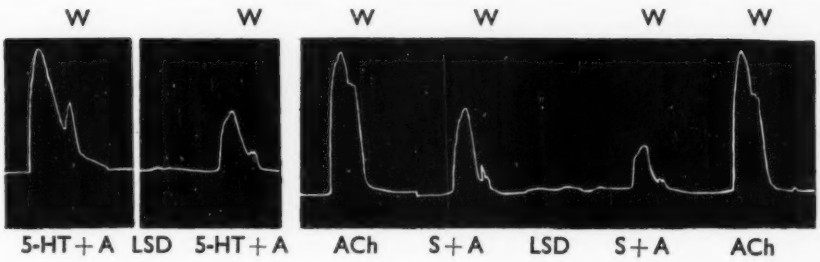

FIG. 1.—Rat's uterus in 25 ml. bath. Antagonism of 5-hydroxytryptamine and of the unidentified substance in nettle stings by lysergic acid diethylamide. A, 0·6 μg. atropine; ACh, 15 μg. acetylcholine; 5-HT, 0·2 μg. 5-hydroxytryptamine; LSD, 0·1 μg. lysergic acid diethylamide; S, 50 nettle stings; W, wash.

washed out. When doses of 5-HT and nettle stings were then given in the presence of 200 μg. tryptamine, together with atropine and mepyramine, the response to 30 nettle stings was reduced by 68% and that to 0.3 μg. 5-HT by 81%.

Experiments with the rat's uterus confirmed this. Desensitization of a preparation by 17 repeated doses of 5-HT reduced the response to 50 nettle stings in the presence of atropine by 53% and the response to 0.4 μg. 5-HT by 51% (Fig. 2). The response to ACh in the absence of atropine was unaffected by desensitization with 5-HT.

Assay of Third Substance.—Third substance was assayed against 5-HT by a matching technique in 7 independent experiments on each of 3 different test preparations (Table I). These assays gave mean values of activity equivalent to 3.43–4.86 ng. 5-HT/sting. A *t* test gave a value of $P=0.2$–0.3 for the difference between the extreme means, which shows that it is not significant.

Paper Chromatography.—In paper chromatograms, both 5-HT and acetone extract of nettle gave a pink spot, turning purple and finally grey, with an R_F value of 0.34. Tryptamine gave a more stable pink spot with an R_F value of 0.61.

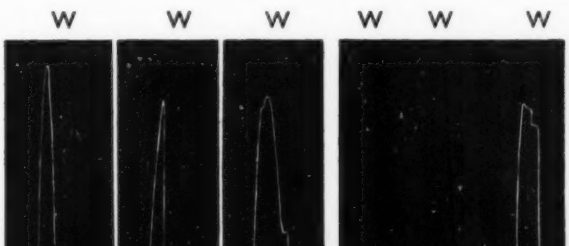

FIG. 2.—Rat's uterus in 25 ml. bath. Desensitization by 5-hydroxytryptamine towards itself and towards the unidentified substance in nettle stings. 5-HT, 0·4 μg. 5-hydroxytryptamine; other abbreviations as in Fig. 1. Between the third and fourth panels, 17 doses of 0·4 μg. 5-HT were given.

TABLE I

THE UNIDENTIFIED SMOOTH-MUSCLE STIMULATING SUBSTANCE IN NETTLE STING ESTIMATED AGAINST 5-HYDROXYTRYPTAMINE (5-HT)

Values obtained on three different preparations *in vitro*

Activity in ng. 5-HT/sting		
Rat's Uterus	Guinea-pig's Ileum	Rat's Colon
2	5	3
2	5	6
8	2	2
4	4	2
7	4	4
8	4	4
3	4	3
Mean .. 4·86	4·00	3·43
S.E. .. 1·03	0·38	0·53

Instability of 5-HT in Nettle Sting Suspensions.—750 ng. quantities of 5-HT were added to a number of fresh suspensions of 50 nettle stings and the 5-HT-activity of one of them estimated immediately on the rat's colon preparation. After the remaining suspensions had stood for various times at room temperature, their activities were estimated in the same way. 5-HT-activity declined sharply on standing, reaching about 10% of its initial value within 30 min. (Fig. 3). After addition of a further 750 ng. 5-HT to suspensions that had stood for 30 min., activity again declined, but more slowly. When 5-HT was added to suspensions of stinge that had previously been boiled, it did not loss activity (Fig. 3).

In another series of experiments the stability of third substance in suspensions of nettle stings, without addition of 5-HT, was examined in the same way. On standing at room temperature, third-substance-activity declined at first sharply and then gradually (Fig. 4). When sting suspensions were boiled dry and the residue taken up in fresh Ringer solution, about 97% of the expected third-substance-activity was lost.

Search for a Fourth Substance.—For the reason discussed below, a possible fourth smooth-muscle

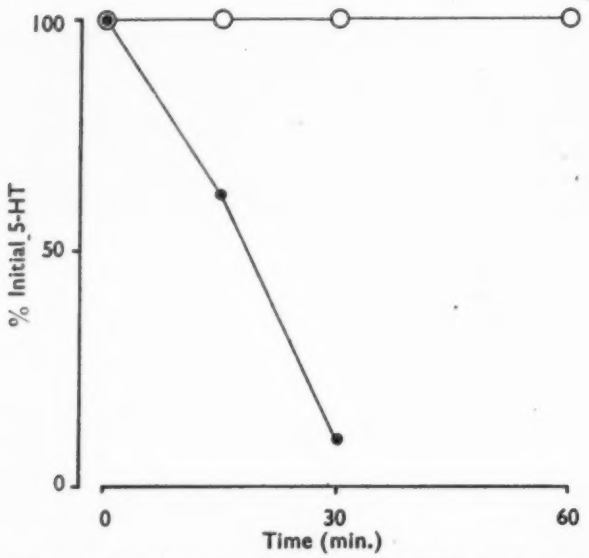

FIG. 3.—Destruction of 5-hydroxytryptamine (5-HT) by nettle stings. 750 ng. 5-HT was added to boiled and unboiled suspensions of 50 stings in Ringer solution and stood at room temperature. 5-HT estimated on rat's colon in presence of atropine. ●—●, sting suspensions unboiled. ○—○, sting suspensions boiled before adding 5-HT.

stimulating substance was sought in nettle stings. Fresh sting suspensions were applied to *in vitro* preparations pre-treated with atropine, LSD and, if necessary, mepyramine. In response to 400 nettle stings, a small contraction of rat's uterus occurred, but this was not greater than might have been expected from a summation of the residual responses to the substances known to be present.

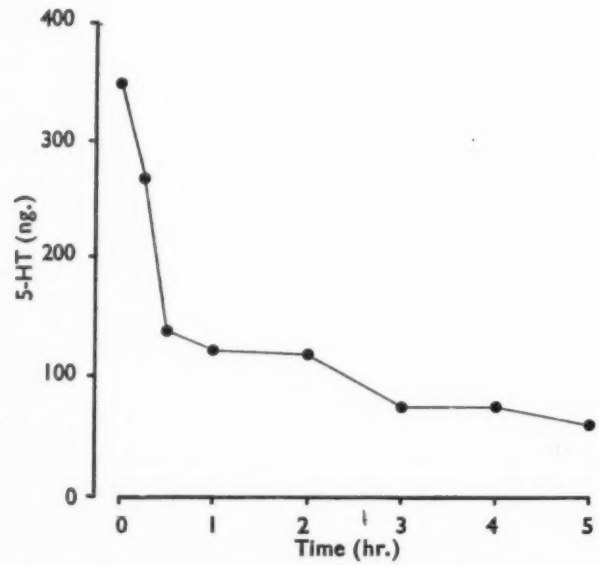

FIG. 4.—Decay of activity of unidentified substance in nettle stings, when suspensions of 50 stings in Ringer solution were stood at room temperature. Activity estimated as 5-hydroxytryptamine (5-HT) on rat's colon in the presence of atropine.

In the presence of antagonists, 400 nettle stings evoked from guinea-pig's ileum a scarcely detectable response, even less than that which a mixture of ACh, histamine and 5-HT, each at the dose expected in 400 stings, produced. On the other hand, a suspension containing 50 μg. dry weight whole wasp venom apparatus evoked from the same preparation, in the presence of the same antagonists, a strong slow contraction, attributable to the kinin which Jaques and Schachter (1954) have shown to be present.

DISCUSSION

The experiments described above showed that the third substance stimulated rat's colon and uterus *in vitro*, was antagonized by LSD, and was inactivated by standing in the presence of nettle stings. Large doses of 5-HT and of tryptamine desensitized *in vitro* preparations towards third substance. These facts indicate the identity of third substance and 5-HT, which quantitative results establish more firmly. On the rat's uterus preparation, LSD antagonized 5-HT and third substance to the same extent, and 5-HT desensitized the preparation towards both substances to the same degree. In quantitative estimates of third substance in terms of 5-HT in three different *in vitro* preparations, the mean values obtained did not differ significantly from one another. The identification of third substance with 5-HT is supported by the detection of 5-HT in paper chromatograms of extracts of nettle leaf and stalk. The detection of 5-HT in nettle adds a second source among plants to that already reported in cowhage by Bowden, Brown, and Batty (1954).

If the average nettle sting contains 7–9 μg. fluid (Emmelin and Feldberg, 1947; Haberlandt, 1886), then 3.4–4.9 ng. 5-HT/sting provides a concentration more than sufficient to evoke pain in human skin (Armstrong, Dry, Keele, and Markham, 1953). It is interesting that Jaques and Schachter (1954) found a very similar concentration of 5-HT (not less than 0.32 mg./g.) in wasp venom.

Although histamine, ACh and 5-HT are more than sufficiently concentrated in nettle sting to account for the triple response and pain, their presence may not explain the pricking or tingling felt even 24 hr. after being stung. This observation suggested that a fourth possible pain-producing substance might exist in nettle. If so, it either does not stimulate smooth-muscle directly or is present in very small amount, since we obtained no evidence of a fourth smooth-muscle stimulating substance in large numbers of nettle stings. The agent which

inactivates 5-HT in unboiled, but not in boiled, suspensions of nettle stings is presumably an enzyme. It is possible that enzymes in nettle sting have pain-producing properties.

Summary

1. Nettle stings contain, besides histamine and acetylcholine, a third active substance which is probably 5-hydroxytryptamine (5-HT). Its identity with 5-HT is suggested by the following tests: the third substance stimulates the rat's uterus *in vitro*; its action is antagonized by lysergic acid diethylamide to the same extent as that of 5-HT; repeated doses of 5-HT desensitize the rat's uterus to the same degree towards the third substance and 5-HT; on paper chromatograms an extract of nettle leaf and stem gave a spot which corresponded in colour and R_F value with 5-HT.

2. *In vitro* estimates gave the following mean values in ng. 5-HT/sting: rat's uterus, 4.86 ± 1.03; guinea-pig's ileum 4.00 ± 0.38; rat's colon, 3.43 ± 0.53.

3. Nettle stings contain an agent, presumably an enzyme, which inactivates 5-HT and the third nettle-sting substance in Ringer solution at room temperature.

4. Evidence of the existence of a fourth substance in nettle sting that stimulates smooth muscle was sought but not found.

We wish to thank Dr. M. Schachter for the gift of dried wasp venom, Miss P. Harknett for technical assistance, and the directors of Allen and Hanburys Ltd. for permission to publish this work. The 5-hydroxytryptamine creatinine sulphate was kindly supplied by Abbott Laboratories and the lysergic acid diethylamide tartrate by Sandoz Products Ltd.

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Brit. J. Pharmacol. (1956), **11**, 190.

THE EFFECT OF ISONIAZID IN EXPERIMENTAL CORNEAL TUBERCULOSIS

BY

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(RECEIVED FEBRUARY 8, 1956)

It has been shown previously that when mice are inoculated intracorneally with *M. tuberculosis* there is a latent period of about 10 days before lesions begin to appear. When such mice are treated with isoniazid (0.3 mg./mouse/day), starting on the day of inoculation, no macroscopic lesions appear during the period of treatment. When the treatment is stopped at the end of 28 days there is again a latent period of some 10 days during which the cornea remains clear; lesions then begin to appear and progress rapidly (Rees and Robson, 1950; Goulding and Robson, 1952).

It would thus seem that the treatment keeps the infection in abeyance but does not eradicate it, and that the development of a macroscopic lesion requires a certain latent period which is of the same order whether it occurs from the time of inoculation or from the cessation of isoniazid treatment.

When, however, larger doses of isoniazid are given during the period of treatment (3.0 mg./mouse/day) withdrawal of the drug is not followed by the development of lesions after a short latent period, and the cornea remains macroscopically clear for a longer time; in some mice lesions then begin to appear and develop to involve much of the cornea. Hence, the more intensive treatment has produced, in some way, an effect which keeps the infection in abeyance for a longer period.

These explanations are, of course, largely descriptive and we do not know what happens in the cornea during the period of isoniazid treatment, or in the latent period preceding the appearance of a macroscopic lesion. We have recently described a technique (Robson and Didcock, 1955) with which it is possible to make detailed observations of cells and bacilli within the cornea at a stage when this tissue appears macroscopically normal. We have used this method to study the phenomena discussed above.

METHODS

Inoculation of the cornea and its examination with the phase contrast microscope have been described previously (Rees and Robson, 1950; Robson and Didcock, 1955). In the present experiments the inoculum consisted of about 5,000 organisms (i.e., a 1/100 dilution of a culture in Dubos medium) of the same strain used in the previous work, unless stated otherwise. Isoniazid was given in the diet, the daily dose being 0.3 mg. or 3.0 mg. in 5 g. of M.R.C. diet 41.

In some experiments an attempt was made to recover the bacilli from the cornea by inoculating it into Kirschner's medium and incubating at 37° C. The details of the procedures are given in the text.

RESULTS

Two experiments were performed. The first investigated the effect of 0.3 mg. of isoniazid, and the second the effect of 3.0 mg.

First Experiment.—This consisted of two main groups of mice—a control group receiving no treatment and a group receiving 0.3 mg. of isoniazid/day from the day of inoculation. In some of these mice, treatment was continued for 16 days and in others for 24 days, in order to study the effect of various periods of treatment.

In a third group of animals isoniazid treatment was started two days before inoculation in order to determine whether pretreatment would affect the course of the ocular lesion. No difference was found between this group and that in which treatment was started on the day of inoculation, and no further reference will be made to this experiment.

In the control group the lesions developed rapidly with the stages previously described: early cellular invasion of the site of inoculation, forming a dense core in which no details could be made out microscopically; and the appearance of organisms in macrophages around the core, with the organisms rapidly multiplying within the

macrophages and destroying them; this coincided with the appearance of a macroscopic lesion in some animals about 5 days after inoculation.

Treatment with isoniazid did not suppress the initial cellular reaction leading to the formation of a dense core, or the appearance of a few bacilli within macrophages around this core. However, as long as treatment was continued (16 or 24 days) the lesion progressed no further; no multiplication of bacilli and no destruction of macrophages were observed. Until the end of treatment, therefore, the lesion presented a relatively quiescent picture, with the bacilli short and almost coccoid in form, in contradistinction to the long, almost filiform organisms present in overgrown macrophages in the absence of treatment. In animals in which treatment was continued for some time we believe that the central core was becoming less dense so that individual cells could now be identified.

In the animals in which treatment was stopped at 16 days, a new macrophage invasion was beginning to occur two days later; in another three days overgrowth and destruction of macrophages by bacilli were seen, and 8 days after cessation of treatment there was complete breakdown with the appearance of a macroscopic lesion. The sequence of events after cessation of treatment was therefore the same as in untreated animals following inoculation.

Second Experiment.—This was on four groups of mice—untreated controls and groups of animals treated respectively for 16, 30, and 50 days with 3.0 mg. of isoniazid/day.

In the untreated controls, lesions developed typically, and by 6 days after inoculation overgrowth of macrophages was occurring, with the appearance of macroscopic lesions in some animals.

When treatment was continued for 50 days, intracellular bacilli (in macrophages) remained visible for the whole period. These organisms rapidly became thin and rather polar, and sometimes almost coccoid in appearance. Occasionally, however, some large forms of bacilli were also seen. Throughout the whole period macrophages and leucocytes were present in the cornea, but at no time were there more than a few bacilli present in any one macrophage. The size and density of the microscopic lesion also gradually decreased. Thus, 48 hr. after inoculation there was a thick core, several hundred micra in diameter, in which no detail could be made out. This core consisted of densely packed cells several layers in thickness. Four days later it was thinning so that some individual cells could be made out, and later still further reduction of the lesion occurred. Thus in one animal, examined 43 days after inoculation, there was only a patch of cells, covering an area roughly 50×25 μ and consisting mainly of macrophages. In another animal, examined 50 days after inoculation, the lesion consisted of a smear of macrophages over an area some 200 μ in diameter. The decrease in the lesion thus involves a reduction in its density as well as in its dimensions. It would thus appear that during the continued treatment with isoniazid many macrophages containing bacilli disappear from the cornea. What happens to the bacteria contained in these macrophages, and particularly whether they leave the cornea dead or alive, is at present unknown. Living bacteria do, however, remain in the cornea throughout the period of treatment, as shown by cultures in Kirschner's medium (containing 1 unit/ml. of penicillin) of corneas removed 33 and 50 days after inoculation. In both experiments the bacteria so obtained were fully sensitive to isoniazid (i.e., to 0.0128 μg./ml.).

When treatment was stopped, either at 16 or 33 days after inoculation, none of the eyes developed macroscopic lesions up to 17 days after cessation of treatment in the first group, and 20 days after cessation of treatment in the second group, when the experiment was terminated. However, changes visible under the microscope did occur, indicating both new cell invasion and some bacterial multiplication.

When treatment was stopped after 16 days, some overgrowth of macrophages by bacilli was beginning 4 days after cessation of treatment, and when treatment had been stopped for 17 days an active-looking lesion was seen consisting of macrophages and leucocytes, though the cornea was still macroscopically clear. The macrophages contained long, almost filamentous bacilli. This cornea was cultured in Kirschner and gave a heavy growth of bacilli fully sensitive to isoniazid.

When treatment was continued for 30 days and then stopped, examination at 13 and 20 days after cessation of treatment showed cell invasion which was particularly striking at the latter period. The cornea, which was macroscopically clear, then contained a dense core 450 μ in diameter, with several satellite micro-lesions around it, consisting of collections of macrophages. Some cellular invasion of the remainder of the cornea was also seen. Small intracellular bacilli were present, but there was at this stage no obvious multiplication, as compared with the eye of an animal in which treatment had been continued for the whole period.

This cornea, too, was cultured and gave a heavy growth of organisms fully sensitive to isoniazid.

When organisms were seen they were mostly in macrophages, as described above. Occasionally a few extracellular organisms were seen in the cornea, both during the period of treatment and after cessation of isoniazid administration.

Difference Between These Two Experiments.—When mice inoculated intracorneally with tuberculosis are treated with 0.3 mg. isoniazid/day and treatment is discontinued, macroscopic lesions appear after a latent period of some 10 days. When the dose of isoniazid is increased to 3.0 mg./day, no macroscopic lesions appear for a longer period after cessation of treatment, though some new cellular invasion can be seen on microscopic examination. There are three possible explanations for the more prolonged suppression of macroscopic lesions with the larger doses of isoniazid:

1. That the number of organisms present in the cornea of animals treated at the higher dose level was depleted during the course of continued isoniazid administration to a greater extent than with the lower dose, so that those left, though undoubtedly alive and capable of multiplication *in vitro*, were no longer sufficiently numerous to initiate rapidly a macroscopic lesion. The experimental data strongly suggest that the number of bacteria in the cornea was markedly decreased.

2. That isoniazid treatment at the higher dose produced an effect on the virulence of the bacilli rendering them unable to produce the usual type of lesion. There is a fair amount of evidence (rather controversial) that bacilli made resistant to isoniazid by being subjected to the prolonged action of the drug become less virulent. The bacilli which failed to produce lesions in the present experiments were indeed not resistant to isoniazid, and there is no suggestion that isoniazid lowers the virulence of the bacilli if it does not increase resistance to the drug.

3. That the presence of bacilli in the cornea during the course of isoniazid treatment produced a condition of immunity so that, after cessation of treatment, their further multiplication with the production of a macroscopic lesion was prevented or slowed down by the immune process. It would then have to be assumed in addition that no such immune process occurred when the treatment was with 0.3 mg. isoniazid/day, but that only bacilli subjected to the higher concentration of the drug were able to initiate the immune reaction. This is not a very likely assumption. Bloch (1955) has given evidence suggesting that BCG bacilli, whose multiplication was arrested by isoniazid administration, were nevertheless capable of conferring immunity in mice.

The Effect of Prolonged Treatment with Isoniazid on the Virulence of Bacilli in the Cornea

One possible explanation for the failure of eyes treated with the larger dose of isoniazid (3 mg./day) to break down soon after cessation of treatment, as compared with eyes treated with a smaller dose of isoniazid (0.3 mg./day), is that the prolonged treatment has reduced the virulence of the organisms. This possibility was investigated in the following way:

The eye of an animal inoculated with a 1 in 100 dilution of a culture of *M. tuberculosis*, and treated for 50 days with 3 mg. isoniazid/day, was removed immediately after cessation of treatment and put into Kirschner's medium containing penicillin (1 unit/ml.). Growth in the tube was first detected some 26 days later and was quite definite in another 7 days. The virulence of this culture was compared with that of the original culture with which the eye had been inoculated, in the following way:

To make the comparison as valid as possible, the two cultures were inoculated at the same time in the same batch of medium and the period of subculture was the same. Viable counts of the two cultures were made by the method of Knox (1955), in order to ensure that the same number of organisms was inoculated, and that any difference in virulence that might appear was not due to difference in bacterial content. Both cultures—the original and the "recovered"—were subcultured in Dubos medium, and various dilutions of the Dubos cultures were then made. These were inoculated intracorneally into mice.

The results of the viable counts were quite clear. The two cultures each contained approximately 50,000,000 organisms/ml. Both cultures were fully and equally sensitive to isoniazid.

The various groups of animals inoculated (each of 6 mice) were observed and lesions noted at various times after inoculation. The average size of the lesions in each group is shown in Table I. The lesions developed at nearly equal rates for the various dilutions in the two groups, strongly suggesting that there was no difference in the virulence of the two cultures, at least as shown by this method. It would thus seem that the failure of the eyes in animals treated with a large dose of isoniazid to break down soon after inoculation is not due to reduced virulence of the organism.

TABLE I

THE DEVELOPMENT OF LESIONS IN MICE INOCULATED WITH VARIOUS DILUTIONS OF ORIGINAL CULTURE (BOVINE) AND BACILLI RECOVERED FROM MICE TREATED WITH ISONIAZID

The values shown represent the averages for the corneal lesions in each group of animals. The size of the lesions was assessed in a semi-quantitative manner as described by Rees and Robson (1950)

	Dilution	No. of Organisms Inoculated	Time After Inoculation (Days)						
			6	7	10	13	17	21	41
Recovered bacilli	1 in 10,000	50	Nil	Nil	Nil	0·4	1·8	1·9	1·7
	1 ,, 1,000	500	,,	,,	,,	0·4	0·7	0·8	0·9
	1 ,, 100	5,000	,,	? Lesions	0·7	1·4	1·8	2·2	2·1
	1 ,, 10	50,000	Lesions	0·5	1·8	2·3	2·5	2·7	2·5
	1 ,, 1	500,000	,,	1·0	2·3	2·4	2·5	2·8	2·7
Original culture	1 in 10,000	50	Nil	Nil	Nil	0·4	1·6	2·1	2·0
	1 ,, 1,000	500	,,	,,	,,	0·3	1·0	1·4	1·3
	1 ,, 100	5,000	,,	0·4	1·4	1·9	2·0	2·1	1·8
	1 ,, 10	50,000	Lesions	0·5	2·1	2·3	2·6	2·7	2·7

The full data on the development of lesions with various dilutions are given in Table I, since such data have not been previously recorded. It is clear that increase in the size of the inoculum can appreciably reduce the latent period preceding the appearance of a macroscopic lesion.

These data also make it possible to calculate the rate of multiplication of the organisms within the cornea, provided the following two assumptions are made: That macroscopic lesions appear when the number of organisms in the cornea has reached a certain level; and that the rate of multiplication of organisms within the cornea is largely independent of the number of organisms present. With an inoculum of 50 organisms there was a latent period of some 13 days whereas with an inoculum 1,000 times larger the latent period was about 6 days. Hence in 7 days the number of organisms had become 1,000 times larger, giving a period of generation of about 17 hr.

Immunity in Mice Following Intracorneal Inoculation

When mice are inoculated intracorneally with *M. tuberculosis* the resulting lesion reaches a maximum at about 30 days, after which it tends to regress somewhat. If at that time the second eye of such animals is inoculated with the same dose of the organism, a second lesion does not develop, even though control eyes develop lesions in the usual way. The inoculation in the first eye has thus led to some type of immunity reaction which protects the animal against a similar second inoculum. This process is now being studied in detail and the results will be published separately. The method has been used to investigate whether mice inoculated intracorneally with *M. tuberculosis* and treated with isoniazid (at 0.3 mg. or 3.0 mg./day) develop any immunity.

The experiment was performed on 6 groups of mice (totalling 90). The groups were those shown in Table II. Groups 1 to 5 were inoculated on day 1 with a 1 in 1,000 dilution of *M. tuberculosis*, i.e., with approximately 500 organisms. Groups 2 and 3 received 0.3 mg. of isoniazid/day from the day of the first inoculation for 36 days, i.e., 4 days

TABLE II

THE EFFECT OF ISONIAZID ON THE DEVELOPMENT OF IMMUNITY TO *M. TUBERCULOSIS* IN MICE

Isoniazid was administered daily to groups 2, 3, 4, and 5 for 36 days; groups 1 and 6 had none. I, intracorneal inoculation with *M. tuberculosis* (about 500 organisms). L, macroscopic lesion. Total no. of mice, 90.

Group	Day 1 (Right Eyes)	Dose of Isoniazid (mg.)	Day 40 (Left Eyes)	Development of Lesions	
				Right Eye	Left Eye
1	I	—	I	L	None
2	I	0·3	Nil	L after treatment	—
3	I	0·3	I	L after treatment	L
4	I	3·0	Nil	L delayed or nil	—
5	I	3·0	I	None	L
6	Nil	—	I	—	L

before the second inoculum. This interval was to ensure that no isoniazid should be present in the body at the time of the second inoculum. Groups 4 and 5 received 3 mg. of isoniazid/day for 36 days. Forty days after the first inoculum groups 1, 3, 5, and 6 received the second inoculum in the left eyes. This consisted of a 1 in 1,000 dilution of a culture of the same strain of *M. tuberculosis*.

The right eyes of the animals in group 1 all developed lesions, which started after the usual latent period of 10–12 days, and progressed to reach a maximum around 30 days. The left eyes of these animals were inoculated 40 days after the

right eyes, and none of them developed a macroscopic lesion (up to 7 weeks after inoculation, i.e., for the total period of observation). Control eyes (group 6) inoculated with the same culture all developed the typical lesions, with the usual latent period. These experiments show quite clearly that inoculation of the right eyes conferred upon the animals a certain degree of immunity which was sufficient to prevent the development of macroscopic lesions in the left eyes. When the second inoculum consisted of the same number of dead bacilli macroscopic lesions also failed to develop, except in one eye in which a tiny spot visible to the naked eye appeared. The microscopic findings in these animals will be described elsewhere.

Animals of groups 2 and 3 received the smaller dose of isoniazid after the right eye inoculation and none had any macroscopic lesions during the period of treatment. After cessation of treatment these eyes broke down, as previously described. The left eyes of the animals in group 3, inoculated 40 days after the right eyes, developed lesions, as did the controls (group 7). Hence the bacilli in the right eyes of these animals, whose multiplication was inhibited by isoniazid, failed to confer any detectable immunity.

The animals of groups 4 and 5 received a larger dose of isoniazid (3 mg./day). Forty days after inoculation the left eyes of group 5 received the second inoculation. The right eyes of group 4 remained clear for 2 months. They were not examined for the subsequent 4 weeks, but then—practically 3 months after inoculation—4 out of 6 had moderate or large lesions of the cornea whereas the other two were still completely clear, and remained clear for the next 2 months—the full period of observation. The right eyes of group 5 remained clear throughout the period of observation, some 3 months after inoculation. The left eyes of group 5, inoculated 40 days after the right ones, developed typical lesions at the usual time, thus showing that here too no immunity detectable by the present method had been conferred by the bacilli in the right eyes.

An interesting result is the difference between the development of lesions in the right eyes (i.e., the eyes receiving the first inoculation) of groups 4 and 5. Whereas no lesions developed in group 5 (in which the left eyes had been inoculated subsequently), 4/6 of the right eyes of group 4 did develop lesions. It would thus appear that the second inoculum in group 5 conferred immunity on these animals which was effective in preventing the breakdown of the right eyes.

Investigation of the Cornea, after Prolonged Treatment with Isoniazid, by Transference to Medium in Vitro

When the cornea is inoculated with some 50 organisms there is a latent period of some 13 days before a macroscopic lesion appears. There is also evidence that under these conditions organisms divide once every 17 hr., and this is, in fact, in agreement with other observations on the rate of multiplication of *M. tuberculosis*. If it be assumed then that only one live organism remains at the end of isoniazid treatment, it would only take about 5 days for this to produce 50 organisms, so that a macroscopic lesion ought to appear some 18 days after cessation of isoniazid action. All this assumes that after cessation of the action of isoniazid the organisms are capable of normal multiplication. In fact we have found that the latent period is more than 24 days and in some animals lesions did not develop even after some two months. This suggests that under these conditions the organisms are not capable of normal multiplication. This does not necessarily imply that their failure to multiply is due to the action of isoniazid, since it has not yet been determined whether a very small number of organisms put into the cornea in the absence of any treatment is capable of initiating a lesion. To throw further light on this question, corneas have been removed from animals at the end of a period of treatment with isoniazid and put into Kirschner's medium to follow the fate of the organisms. The following results have so far been obtained:

1. The cornea of a mouse, treated for 33 days with 0.3 mg. isoniazid/day, was removed under sterile precautions, washed with sterile saline to remove any isoniazid present in it, and examined microscopically; it was then transferred to Kirschner's medium containing 20 units/ml. of penicillin and 25 μg./ml. of chloramphenicol. These antibiotics were added, as in some previous experiments secondary infection was troublesome.

After 2 days in Kirschner the cornea was removed and examined under the phase contrast microscope. There appeared to be no change. After another 3 days in Kirschner it was examined again and was found to contain large numbers of bacilli with signs of early cord formation. The bacilli were associated with cells or cellular debris and large numbers were also found in nerves, either free or in cells. It is obvious, therefore, that under these conditions a tremendous bacterial multiplication had occurred within 5 days.

In another experiment two mice were treated for 57 days with 0.3 mg. of isoniazid/day and the

corneas were then removed, and transferred to Kirschner's medium with 20 units of penicillin/ml. and no chloramphenicol, since chloramphenicol has some tuberculostatic action. Before transferring to Kirschner, both these corneas (C and D) were examined under the phase contrast microscope. Both were clear on naked eye examination. C contained an impenetrable lesion some 200 μ in diameter, with a few macrophages and corneal corpuscles around it. No bacilli were seen. D contained a core consisting of a smear of macrophages and corneal corpuscles. A few bacilli were seen both extracellularly and intracellularly.

After 4 days in Kirschner, D was examined again ; it contained large numbers of bacilli in the area of the lesion, which had not extended. There was no cord formation. C was examined after 6 days in Kirschner ; it contained a few bacilli. C was again examined after 8 and after 12 days in Kirschner, and again a few bacilli were found, perhaps fewer than after 6 days. Hence marked development had occurred in D and some growth, though not marked, in C.

2. A cornea removed from a mouse after 33 days' treatment with isoniazid (3 mg./day) was washed to remove the isoniazid and then put into Kirschner with penicillin and chloramphenicol. The cornea was examined 2, 4, 6, 10, 16, and 23 days later. Organisms were seen at these various stages, but no evidence of multiplication was obtained. Several organisms forming a definite pattern were seen and photographed ; 4 days later the same field was accurately identified and again photographed—there was no evidence that the organisms had changed or multiplied.

The cornea was put back into Kirschner without chloramphenicol, but secondary infection soon appeared and the preparation was discarded.

In another experiment two mice were treated with 3 mg. isoniazid/day for 57 days and the corneas (C and D) were then removed and examined under the phase contrast microscope. The lesion in C consisted essentially of a smear of macrophages, corneal corpuscles, and some dots, but no bacilli were definitely identified, though a few coccoid objects might have been bacilli. D had a dense lesion in which some cells could still be made out and also contained blood vessels in which rouleaux of red blood cells could be seen. There were macrophages and corneal corpuscles around the lesion. No organisms were identified. C was again examined after 4 days in Kirschner containing penicillin but no chloramphenicol. No obvious change had occurred. D was examined after 6 and 8 days in Kirschner, and both C and D were examined after 12 days in Kirschner. At none of these examinations were any bacilli seen. Hence in the corneas of these animals no growth had occurred in Kirschner's medium.

Discussion

One of the most important problems in the chemotherapeutic treatment of tuberculosis is the frequent failure of drugs to eradicate the organisms from the body. Though such treatment is, as a rule, strikingly successful in the control of signs and symptoms, the disease relapses in many patients at various times after cessation of drug administration. The present experiments help to throw some light on what is happening in such lesions, though they are not concerned with drug resistance, which is an important factor in the control of human disease.

In the first place they show that prolonged treatment with isoniazid, though it checks the multiplication of the bacilli, does not kill all of them. They remain visible in macrophages, mostly as small, thin bacilli, presumably a resting form ; and on cessation of treatment they can start multiplying again, as is demonstrated by growth in Kirschner's medium and also by the subsequent development of typical lesions in the cornea. Another point of interest is that, as the duration of isoniazid therapy increases, the extent of the microscopic corneal lesion gradually decreases ; this involves not only a decrease in the diameter and thickness of the aggregate of cells which make up the lesion but also a decrease in the density of the cell population, so that as time elapses cells become individually visible, whereas at the beginning of treatment the dense packing of the cells within the lesion made it impossible to see any detail under the phase contrast microscope. What happens to the cells which disappear from the lesion and, more important, to the bacilli which they presumably contain ? The duration of life of such cells is in all probability quite limited and thus they must break down, liberating the bacilli which they contain. Such bacilli may then (1) die and disintegrate and this may of course also happen while the bacilli are still within cells ; (2) be carried away to other parts of the body where they may be dealt with ; or (3) be taken up by macrophages within the lesion, thus increasing the population of bacilli per cell. This third possibility is not supported by what we have seen by the examination of lesions under the phase contrast microscope, for there was no suggestion of any increase in the number of bacilli per cell.

We realize that the maximum period for which we have treated the animals in these experiments—50 days—is short as compared with the many months of chemotherapy now usual in human tuberculosis. It would obviously be of interest to determine what happens to corneal lesions subjected to more prolonged action of drugs. It may well be that the gradual attrition of a lesion, with progressive diminution of cell population and bacterial content, represents the mechanism by which the body conquers the disease under the protection of chemotherapy.

The delayed breakdown, or sometimes even the absence of breakdown, of macroscopic lesions after prolonged chemotherapy with large doses of isoniazid, as compared with the regular breakdown after smaller doses of isoniazid, represents another problem. Similar findings have previously been observed with combined chemotherapy of corneal infections as, for example, with isoniazid and streptomycin (Goulding and Robson, 1952) and with streptomycin and a thiosemicarbazone (Rees and Robson, 1951); in these experiments breakdown sometimes occurred many weeks after cessation of treatment. These findings too have their clinical counterpart in the relapse which may occur in human tuberculosis after cessation of treatment with combinations of drugs.

A possible explanation for such a delayed breakdown is that the prolonged treatment has decreased the virulence of the organisms within the lesions. The present experiments have shown that this is not so within the limits of the very special conditions under which they were carried out: they do show that organisms recovered from the cornea of an animal subjected to prolonged administration of isoniazid in large doses are not less capable of producing corneal lesions than are the organisms originally inoculated.

The question as to whether organisms in the cornea subjected to the action of isoniazid (at both doses) can lead to the development of an immunity reaction was also investigated. It was shown quite clearly that no immunity could be demonstrated in the other eye of such animals, even though a striking immunity can be shown in the second eye when the lesion in the first eye is allowed to develop unhampered by any drug action. This question too is of practical importance, and contradictory results have been obtained by different investigations (see Bloch, 1955). More recently Palmer and Ferebee (1955) have found that, when guinea-pigs are infected with *M. tuberculosis* and treated with isoniazid in doses which protect the animals, such a controlled infection does confer some degree of resistance to a subsequent challenge. It may well be that the size of the original inoculum and the exact extent to which isoniazid controls its multiplication determine whether any immunity reaction occurs. This problem requires further investigation, particularly since it has been suggested that isoniazid should be used prophylactically in man, and that such use would not interfere with the development of immunity to tuberculosis.

The likeliest explanation for the delayed breakdown after prolonged treatment with large doses of isoniazid is that the number of organisms in the cornea has been so depleted that they no longer represent a sufficient inoculum to start immediate multiplication, or, in some cases, even to lead to the development of a lesion. This question will not be further discussed at present since it is proposed to study the effect of inocula smaller than 50 organisms (the smallest used in the present experiments) to determine whether they are capable of initiating a lesion in the cornea, and, if so, with what latent period. It is interesting, however, that the experiments in which the cornea was transferred to an artificial medium *in vitro* (Kirschner's medium) revealed differences between the corneas of animals treated respectively with 0.3 mg. and 3.0 mg. of isoniazid/day, similar to those demonstrated in the corneas which remained in the living animals. There are a number of snags in the interpretation of such *in vitro* experiments which are discussed in the masterly review by Brieger (1951). We are performing further experiments on the fate of organisms in the cornea transferred to various media outside the body.

Summary

1. The changes which occur in the corneas of mice inoculated intracorneally with *M. tuberculosis* and treated orally with isoniazid have been studied by means of the phase contrast microscope. The treatment does not interfere with the initial cellular invasion of the cornea, but organisms do not multiply appreciably within the cells during the period of drug administration. When the dose of isoniazid is 0.3 mg./mouse/day, cessation of treatment is followed by bacterial multiplication and the appearance of a macroscopic lesion within a period of some 10 days, i.e., the same as the latent period before the appearance of a lesion in the untreated animals. When the dose of isoniazid is 3.0 mg./day the lesion appears a good deal longer after cessation of treatment.

2. Possible explanations for this difference have been investigated. It has been shown: (*a*) That treatment with the larger dose of isoniazid for

50 days has not affected the virulence of the organisms remaining in the cornea after this time. (*b*) That when the development of a lesion is prevented by the administration of isoniazid (in either dose), inoculation of the second eye leads to the development of a normal lesion. Such a lesion does not develop in the second eye when the first infection remains untreated. Hence the isoniazid-controlled infection of the first eye failed to confer any systemic immunity. (*c*) That when the cornea of a mouse treated with 0.3 mg. isoniazid/day is removed from the animal and put into Kirschner's medium rapid multiplication of the organisms *in situ* can be demonstrated. When a similar experiment is performed with a cornea of an animal treated with the larger dose of isoniazid such rapid multiplication is not observed.

3. The relevance of these findings to the chemotherapy of human tuberculosis, and in particular to the reappearance of active lesions after prolonged chemotherapy, is discussed.

We are greatly indebted to Professor R. Knox for his advice during the course of this work; to Professor Knox and Mr. R. C. Woodroffe for the bacterial counts in the virulence experiment; to Dr. M. Naguib for inoculating a number of animals; and to the Medical Research Council for defraying in part the expenses of this investigation.

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THE GANGLION-BLOCKING PROPERTIES OF HEXAMETHYLENE BISDIALKYLSULPHONIUM SALTS

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(RECEIVED FEBRUARY 8, 1956)

The pharmacological properties of tertiary sulphonium salts appear to be similar to those of quaternary ammonium salts. The trimethylsulphonium ion, $Me_3\overset{+}{S}$, resembles the tetramethylammonioum ion, $Me_4\overset{+}{N}$, in its ability to paralyse the frog sartorius preparation (Ing and Wright, 1933): decamethylene bisdimethylsulphonium $Me_2\overset{+}{S}-(CH_2)_{10}-\overset{+}{S}Me_2$, resembles decamethonium, $Me_3\overset{+}{N}-(CH_2)_{10}-\overset{+}{N}Me_3$, in its neuromuscular blocking properties (Walker, 1950): acetoxyethyldimethylsulphonium, $CH_3COOCH_2CH_2\overset{+}{S}Me_2$, resembles acetylcholine, $CH_3COOCH_2CH_2\overset{+}{N}Me_3$, in its effects on cat's blood pressure, rabbit's intestine, frog's heart and frog's rectus (Ing, Kordik, and Tudor Williams, 1952). The resemblance, however, is only qualitative; decamethylene bisdimethylsulphonium has only about 6% of the neuromuscular blocking activity of decamethonium by the rabbit head-drop test: acetoxyethyldimethylsulphonium has only between 2% and 6% of the activity of acetylcholine on the preparations listed above.

Ing, Kordik, and Tudor Williams suggested that there might be some connexion between the activity of onium analogues of acetylcholine and the size of their cationic head—a molecule with a large head, such as sulphonium or arsonium, might be less able to fit the receptor group normally holding the quaternary ammonium group in acetylcholine.

Another possibility is that the low activity of the tertiary sulphonium salts may be because they have only two methyl groups attached to the onium atom: the other compounds examined have three methyl groups attached to the onium atom. We noted that, in the results obtained by Ing *et al.*, the phosphonium analogue of acetylcholine ($CH_3COOCH_2CH_2\overset{+}{P}Me_3$; C–P bond, 1.87Å) was appreciably more active than the sulphonium analogue ($CH_3COOCH_2CH_2\overset{+}{S}Me_2$; C–S bond, 1.82Å; the C–S–C bond angle in sulphonium salts does not seem to be known.) Further, the activity of the sulphonium analogue of acetylcholine was about the same as that of acetoxyethyldimethylamine,

$$CH_3COOCH_2CH_2\overset{+}{N}Me_2H.$$

It seemed to us, therefore, that tertiary sulphonium salts should be compared with salts of tertiary amines (provided these are in the ionized form in the conditions of the tests) rather than with quaternary salts.

To test this idea, we have compared the ganglion-blocking properties of hexamethonium, hexamethylene bisdimethylsulphonium, and of salts of hexamethylene bisdimethylamine. We have also examined the effects of replacing methyl groups in these compounds by ethyl groups. The variation of activity with the number of groups thus substituted in the hexamethonium molecule is already known (Wien and Mason, 1951). We were interested to see if there was a similar variation in activity in the analogous series of bistertiary amine dihydrobromides:

$Me_2\overset{+}{H}N.(CH_2)_6.\overset{+}{N}HMe_2$, $2Br^-$, hexamethylene bisdimethylamine dihydrobromide,

$MeEt\overset{+}{H}N.(CH_2)_6.\overset{+}{N}HEtMe$, $2Br^-$, hexamethylene bisethylmethylamine dihydrobromide, and

$Et_2\overset{+}{H}N.(CH_2)_6.\overset{+}{N}HEt_2$, $2Br^-$, hexamethylene bisdiethylamine dihydrobromide,

and if the variation in the analogous series of sulphonium salts,

$Me_2\overset{+}{S}(CH_2)_6\overset{+}{S}Me_2$, $2I^-$, hexamethylene bisdimethylsulphonium diiodide,

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$MeEt\overset{+}{S}.(CH_2)_6\overset{+}{S}EtMe$, $2I^-$, hexamethylene bisethylmethylsulphonium diiodide, and

$Et_2\overset{+}{S}.(CH_2)_6\overset{+}{S}Et_2$, $2I^-$, hexamethylene bisdiethylsulphonium diiodide,

resembled the variation in the hexamethonium series or that in the series of bistertiary amine dihydrobromides.

METHODS

The ganglion-blocking activity of the compounds relative to hexamethonium was determined in cats anaesthetized with pentobarbitone sodium (30 mg./kg.). The sympathetic trunk in one side of the neck, carefully dissected free from the vagus nerve and other tissues, was tied and cut. The peripheral end was laid across a pair of electrodes connected to the output of a square wave stimulator. The nictitating membrane was attached to a lever (18:1 magnification) by a thread running over a light pulley. Records of the movements of the lever and of the blood pressure (from a mercury manometer attached to a femoral artery) were made simultaneously on a smoked paper. All drugs were injected intravenously through a cannula in a femoral vein and washed into the circulation with 2–3 ml. of saline.

The preganglionic sympathetic trunk was stimulated for 10 sec. by the application of a series of maximal shocks at a rate of 20 shocks/sec. During this stimulation the nictitating membrane contracted at a fast rate for the first few seconds and then more slowly. At the end of ten seconds the rate of contraction was negligible. When the stimulation ceased, the membrane slowly relaxed and returned to its original position within 40–70 sec. The preparation was then ready for further stimulation: usually, therefore, the nerve was stimulated for 10 seconds every minute.

When a series of contractions of similar height had been obtained a dose of hexamethonium was given intravenously to test the sensitivity of the preparation. It was usually found that 200 μg. hexamethonium (as ion) reduced the contraction of the nictitating membrane by about 20–30%. Thereafter, a dose of the test drug was given between doses of hexamethonium which produced smaller and larger effects. Three or four different doses of hexamethonium were used, so that at the end of the experiment a graph could be obtained of the logarithm of the dose plotted against the effect. From this graph it was possible to calculate the dose of hexamethonium which would produce an effect equivalent to that produced by the dose of test drug, and hence the number of molecules of the test drug required to produce the same effect as one molecule of hexamethonium. The relative activities of the test drug and of hexamethonium are thus expressed as an equipotent molar ratio. Graphs of the logarithm of the dose plotted against the effect were also obtained for the most potent drugs in each series, and these were found to be parallel to the graph for hexamethonium.

RESULTS

These are summarized in Table I. The figures indicate the number of molecules of a compound (± the standard error) required to produce the same effect as one molecule of hexamethonium. A high number indicates a low activity.

TABLE I

BLOCKING EFFECTS OF COMPOUNDS ON THE SUPERIOR CERVICAL GANGLION OF THE CAT

Activities are expressed as the number of molecules of the compound (± the standard error) required to produce the same effect as one molecule of hexamethonium—that is, as equipotent molar ratios. The larger the ratio, the lower the activity.

Compound	No. of Expts.	Mean Equipotent Molar Ratio ± Standard Error
Hexamethylene bisdimethylsulphonium iodide	4	20·2±1·8
,, bisethylmethylsulphonium iodide	5	3·5±0·2
,, bisdiethylsulphonium iodide ..	6	1·9±0·2
,, bisdimethylamine dihydrobromide	16	12·0±1·4
Hexamethylene bisethylmethylamine dihydrobromide	13	8·2±0·6
Hexamethylene bisdiethylamine dihydrobromide	13	7·1±0·5
Hexamethonium		1·0

In doses large enough to abolish completely the response of the nictitating membrane to preganglionic stimulation, none of the compounds reduced the contractions produced either by adrenaline or by postganglionic stimulation. Nor did any of the compounds stimulate the ganglion, when given either in small doses or in doses large enough to cause complete block.

Freshly made solutions in distilled water of the salts of the tertiary amines were slightly acid, but only a very small amount of alkali was needed to bring the solutions to *p*H 7.6. This amount corresponded to less than 5 milliequivalents of the bisdiethyl compound and to less than 25 milliequivalents of the other two compounds. In solution at *p*H 7.6, then, not more than 0.5% hydrolysis of the dihydrobromide of the bisdiethyl compound had occurred, and not more than 2.5% hydrolysis of the other compounds. It would seem reasonable to assume, therefore, that it is the ionized forms of these compounds which are active.

The duration of action of most of the substances was less than that of hexamethonium. The bisdimethyl compounds in both series had the briefest action; after the intravenous injection the effects lasted about half as long as those of an equipotent dose of hexamethonium. The bismethylethyl and

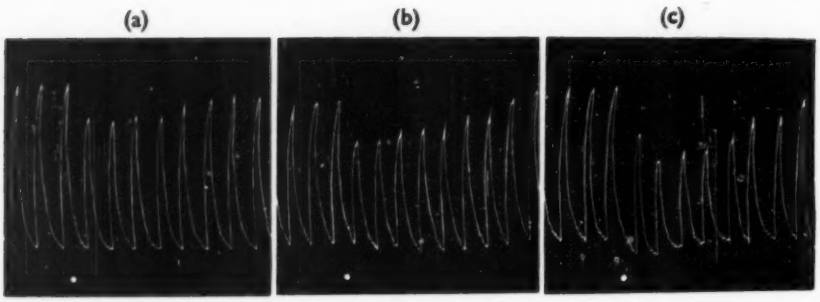

FIG. 1.—Cat, ♀, 2·2 kg., pentobarbitone sodium 30 mg./kg. Contractions of nictitating membrane. Sympathetic trunk stimulated at 20/sec. with supramaximal pulses for 10 sec. every minute. The figure shows the effects of (a), 250 μg. hexamethonium bromide; (b), 2·5 mg. hexamethylene bisdiethylamine dihydrobromide; and (c), 1 mg. hexamethylene bisdiethylsulphonium iodide, all given intravenously. Doses are expressed as base.

bisdiethyl compounds were longer acting (Fig. 1): in fact the duration of action seemed to vary with the potency, the most potent compound, hexamethylene bisdiethylsulphonium, being the longest acting and producing effects which lasted about the same time as those of a comparable dose of hexamethonium.

DISCUSSION

The results confirm our impression that tertiary sulphonium compounds are pharmacologically similar to tertiary ammonium compounds—that is, to the salts of tertiary amines (provided these are not extensively hydrolysed at *p*H 7.6). They also strengthen the argument that the low activity of the sulphur analogue of acetylcholine, acetoxyethyldimethylsulphonium, may be ascribed to its possession of only two methyl groups attached to the onium atom. The beneficial effects on activity of replacing all the methyl groups in the sulphonium and tertiary ammonium compounds by ethyl groups is rather surprising, in view of the effects of these changes in the hexamethonium molecule. However, one is reminded of the fallacy of regarding particular groups in a cationic head as being beneficial to, or detrimental to, activity. This fallacy was thoroughly exposed by the work of Ing and Wright (1931, 1933; review by Ing, 1936) on simple onium salts. They showed that, although many onium compounds containing ethyl groups were much less active blocking agents on the frog sartorius preparation than their methyl analogues, this was not invariably true. The ethyl group was not intrinsically detrimental to activity—the size of the central onium atom and the nature of the other groups attached to it had to be considered. It would seem quite possible, therefore, that further search among sulphonium or tertiary ammonium compounds might be rewarding.

CHEMICAL SECTION

Analyses are by the Huffman Microanalytical Laboratories, Colorado: m.p.s are uncorrected.

Preparation of hexamethylene bisdimethyl, bisethylmethyl, and bisdiethyl sulphonium diiodides.—Hexamethylene diiodide, an equal volume of ethanol and a large excess of the appropriate sulphide were sealed up and left at room temperature (approximately 30° C.). After a few days, crystals of the sulphonium salt began to form, and at the end of from two to six weeks the tube was opened and the salt filtered off. The substances were recrystallized from mixtures of methanol and ethanol, care being taken to avoid heating them above 60° because they decompose rapidly when heated.

Hexamethylene bisdimethylsulphonium diiodide melted at 146–7°(dec.) in a sealed tube. Found: C, 26.0; H., 5.27; I^-, 54.7. $C_{10}H_{21}S_2I_2$ requires C, 26.0; H, 5.25; I^-, 55.0%.

Hexamethylene bisethylmethylsulphonium diiodide melted at 83–4° in a sealed tube. Found: C, 31.2; H, 6.01; I^-, 48.8. $C_{12}H_{28}S_2I_2$ requires C, 29.4; H, 5.77; I^-, 51.9; $C_{12}H_{28}S_2I_2$, 0.75 EtOH, requires C, 30.9; H, 6.25; I^-, 48.4%.

Hexamethylene bisdiethylsulphonium diiodide melted at 151–2° (dec.) in a sealed tube. Found: C, 32.6; H, 6.34; I^-, 48.9. $C_{14}H_{32}S_2I_2$ requires C, 32.4; H, 6.30; I^-, 49.0%.

Hexamethylene bisethylmethylsulphonium diiodide appeared to contain ethanol of crystallization. It was not possible to determine this directly because the compound decomposed when heated. The ultra-violet absorption spectra of the sulphonium compounds in water show a maximum at 226–227 mμ. The absorption is apparently due entirely to the iodide ion present, because the absorption spectrum of sodium iodide in water has a peak at exactly the same wavelength. The values of $\log\varepsilon$ (molar) were 4.38 and 4.39 for the bisdimethyl and bisdiethyl compounds respectively. From the absorption of a solution of the bisethylmethyl compound of known concentration, it was therefore possible to obtain two estimates of the molecular weight of the compound. The values, 526 and 513, agreed more closely with the theoretical value (525) for the formula with 0.75 molecules of ethanol of crystallization than with that (490) for the formula without alcohol.

Preparation of hexamethylene bisdimethyl, bisethylmethyl, and bisdiethylamine dihydrobromides.—Hexamethylene dibromide, dissolved in ethanol, was

refluxed with an excess of the appropriate secondary base. After several hours the ethanol and unreacted base were distilled off under reduced pressure and the solid residue treated with strong sodium hydroxide solution. The free base liberated was extracted with ether and the ether extract dried with solid sodium hydroxide, filtered, and the ether distilled off. The residue was treated with an excess of concentrated hydrobromic acid and the dihydrobromide recrystallized from mixtures of ethanol and methanol.

Hexamethylene bisdimethylamine dihydrobromide melted at 223°. Found : Br^-, 47.8. $C_{10}H_{26}N_2Br_2$ requires Br^-, 47.9%.

Hexamethylene bisethylmethylamine dihydrobromide melted at 213–4°. Found : Br^-, 44.1. $C_{12}H_{30}N_2Br_2$ requires Br^-, 44.2%.

Hexamethylene bisdiethylamine dihydrobromide melted at 267°. Found : Br^-, 41.1. $C_{14}H_{34}N_2Br_2$ requires Br^-, 41.0%.

Summary

1. Hexamethylene bisdimethyl, bisethylmethyl, and bisdiethylsulphonium iodides have been prepared and tested on the superior cervical ganglion of the cat.

2. The ganglion-blocking activity was greatest in the bisdiethyl compound and least in the bisdimethyl compound. On a molecular basis, hexamethylene bisdiethylsulphonium iodide has about half the ganglion-blocking activity of hexamethonium on the preparation.

3. The analogous tertiary amine dihydrobromides, hexamethylene bisdimethyl, bisethylmethyl, and bisdiethyl amine dihydrobromides, also had ganglion-blocking properties. The bisdiethyl compound was again the most active and the bisdimethyl compound the least.

4. The bearing of these results on the difference between the properties of acetylcholine and acetoxyethyldimethylsulphonium is discussed.

We wish to thank Dr. Arnold D. Welch for his encouragement and interest in this work and Miss Yvonne Johnson and Miss Ruth Wolf for their technical assistance. One of us (R. B. B.) gratefully acknowledges the award by Yale University of an Alexander Brown Coxe Fellowship. The work was supported, in part, by a grant from the National Institute of Health, U.S. Public Health Service.

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THE RELEASE OF 5-HYDROXYTRYPTAMINE BY HISTAMINE LIBERATORS

BY

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(RECEIVED FEBRUARY 9, 1956)

The experiments to be described show that histamine liberators release 5-hydroxytryptamine (5-HT), at least in the rat, in addition to histamine. It is generally supposed that histamine is stored in and released from the mast cells and it seems probable that these cells are also the source of the release of 5-HT. The experiments were therefore designed to find out whether the mast cells were the source of the 5-HT released by histamine liberators. Since the experiments have been concluded Benditt, Wong, Arose, and Roeper (1955) have reported that 5-HT is a natural constituent of the mast cells of rats.

METHODS

The effect of histamine liberators was examined on rat, rabbit, cat, and dog tissues perfused with oxygenated Locke solution.

Perfusion of Rat Tissues.—The rats were anaesthetized with sodium pentobarbitone (40 mg./kg. i.p.) and the following tissues were perfused : the hindquarters, one hind limb, the gastrocnemius muscle, and the skin flaps of the hind limb.

The hindquarters of the rat were perfused, as described by Feldberg and Mongar (1954), through the abdominal aorta, and the effluent was collected from the vena cava. For perfusion of the hind limb two methods were used : in the first, the perfusion fluid was introduced directly through the femoral artery, and the limb, which was ligated as high as possible, was severed from the animal and transferred to a paraffin bath at 37° C. ; in the second, a long polythene arterial cannula was introduced through the abdominal aorta and passed into the femoral artery, the limb being perfused *in situ* and kept warm with cotton-wool pads soaked in warm paraffin. In both methods the effluent was collected from the femoral vein. In some of these experiments on the perfused limb the skin was carefully removed using a thermocautery, after ligating and cutting the main saphenous vessels.

The gastrocnemius muscle was perfused through the femoral artery and the effluent collected from the femoral vein. The muscle was prepared as described for the cat gastrocnemius by Feldberg and Paton (1951).

The skin perfused was the area supplied by the superficial epigastric artery. In the rat this artery supplies an area of skin on the upper hind leg and part of the adjacent abdomen and flank. In the first experiments the method described by Feldberg and Paton (1951) for the cat's skin flap was used. This method has the disadvantage that in the rat the skin flap when removed from the limb becomes very small owing to strong contraction. In later experiments therefore the area of skin was neither removed from the leg nor cut, but its blood circulation was separated from that of neighbouring skin areas by cauterizing the circumference of the area. The femoral and saphenous vessels were then ligated beyond the origin of the saphenous vessels in the popliteal region. For this purpose the skin was cut with a thermocautery from the popliteal region to the ankle. The perfusion fluid was introduced through a cannula in the femoral artery after all side branches between the abdominal aorta and the origin of the superficial epigastric were ligated. The venous effluent was collected from the femoral vein, all branches from the muscles being ligated. Evans blue, injected intra-arterially at the end of the perfusion, marked the area of skin that was perfused. Evans blue was also injected at the end of all other perfusion experiments of the rat tissues in order to estimate the extent of perfusion.

Perfusion of Tissues from Species Other Than the Rat.—Rabbits were anaesthetized with urethane (1.25 g./kg. i.v.), cats and dogs with sodium pentobarbitone (40 mg./kg. i.p.). In dogs the limbs, in rabbits the hindquarters, and in cats the hindquarters, the gastrocnemius muscles and skin flaps from the hind limb, were perfused. The perfusion of the hindquarters was the same as that described for rats. The skin flaps and gastrocnemius muscles in cats were perfused as described by Feldberg and Paton (1951). The hind limb of the dog was perfused through the femoral artery and the effluent collected from the femoral vein. The limb was ligated, severed from the animal, and kept warm with cotton-wool pads soaked in warm paraffin.

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Assay of Perfusate.—Samples of perfusate were tested on the atropinized guinea-pig's ileum, and on the atropinized colon and uterus of rat, suspended in a 15 ml. bath. The suspension fluid for the guinea-pig's ileum was Mg free Tyrode solution and for the rat's tissues de Jalon solution. Bath temperature for the guinea-pig's ileum was 34° C., for the rat's colon 24° C., and for the rat's uterus 29–30° C. Histamine was assayed on the guinea-pig's ileum and 5-HT on the atropinized rat's colon; all values refer to base.

Chromatographic Analysis.—The 5-HT in the perfusate was identified by paper chromatography. The substance was extracted three times with acetone (5 ml.) from freeze dried perfusate (50 ml.). The extract was evaporated in a current of air to a small volume, transferred to a Whatman No. 1 filter paper and chromatographed using butanol-acetic acid-water (5:1:4). Identification was by the method of Jepson and Stevens (1953), which consists of spraying the strip with ninhydrin reagent, heating at 90–100° C. for 2 to 3 min., and examining under ultra-violet light for fluorescence. Comparison was made with a sample of 5-hydroxytryptamine creatinine sulphate.

RESULTS

Perfusion of the Rat's Hindquarters

Perfusate collected from the hindquarters of the rat after injection of compound 48/80 contracted not only the guinea-pig's ileum but also the atropinized rat's uterus which is insensitive to histamine (Fig. 1d). The contraction of the rat's uterus was not due to the presence in the perfusate of smooth muscle contracting polypeptides such as bradykinin or substance P, as shown by the following experiment: Both bradykinin and substance P are readily destroyed by chymotrypsin, whereas the activity in the perfusate remained unaffected after incubation with chymotrypsin under similar conditions. The effect of treatment with chymotrypsin on bradykinin and perfusate is illustrated in Fig. 1. On the other hand the contractions of the rat's

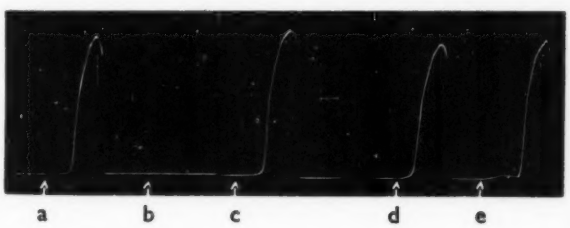

FIG. 1.—Rat uterus suspended in 15 ml. de Jalon solution; 2 μg. atropine added at each washing. At (a) and (c) response to 5 μg. bradykinin and at (b) 5 μg. bradykinin after incubation with 100 μg. chymotrypsin at 37° C. for 5 min. Responses to 0·8 ml. perfusate alone at (d) and after incubation with 100 μg. chymotrypsin at (e).

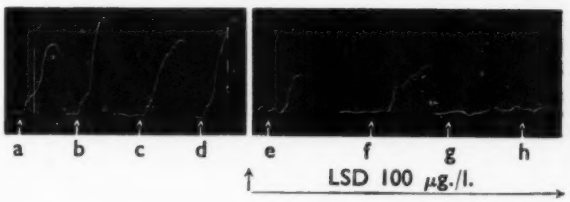

FIG. 2.—Rat colon suspended in 15 ml. of de Jalon solution at 24° C. After each washing 2 μg. atropine added. Additions to the organ bath were made at 5 min. intervals. At (a) 0·03 μg. and at (d), (e), and (g) 0·04 μg. 5-HT creatinine sulphate; at (c) 0·4 ml. and at (b), (f), and (h) 0·5 ml. of 1:10 perfusate collected after 100 μg. 48/80. During (e), (f), (g), and (h) 1·5 μg. lysergic acid diethylamide added at each washing.

uterus produced by the perfusate were antagonized by dihydroergotamine and by lysergic acid diethylamide, which are antagonists of 5-HT. In addition the perfusate collected after 48/80 also contracted the isolated rat's colon, which is insensitive to histamine but sensitive to 5-HT, and again the contractions produced by perfusate were antagonized by lysergic acid diethylamide. In the experiment of Fig. 2 the responses of the rat colon to equiactive doses of 5-HT and perfusate were reduced to the same extent, or abolished, in the presence of lysergic acid diethylamide 100 μg./l. Dihydroergotamine (100 μg./l.) had the same effect.

These results with dihydroergotamine and lysergic acid diethylamide suggested that the active substance in the perfusate was 5-HT. This was confirmed by chromatographic analysis. In these experiments the fluorescence characteristics described by Jepson and Stevens for 5-HT were fulfilled except that the activity in the perfusate flowed slightly faster than 5-HT. The R_F for the perfusate was 0.49, and for the synthetic 5-HT 0.43. This difference was not due to the synthetic drug being the creatinine sulphate and the liberated 5-HT a different salt, but to the presence of some interfering substances in the perfusate. This is shown by the following experiment: Samples of perfusate collected before (sample A) and after (sample B) injection of 100 μg. 48/80 were assayed against 5-HT. Sample A contained the equivalent of 0.1 μg. and sample B the equivalent of 5 μg. of 5-HT. Before extraction with acetone 5 μg. of synthetic 5-HT was added to sample A and both samples prepared for chromatography. In this experiment the R_F value for 5-HT in sample A was 0.50 and in sample B 0.49. One part of sample A chromatographed without adding 5-HT gave no fluorescent spot.

The biological and chemical tests thus both allow the conclusion that the substance contracting the smooth muscle tissues of the rat was 5-HT.

Comparison of the Release of Histamine and 5-HT.—The effluent collected from the rat's hindquarters before injection of 48/80 usually caused no contraction of the atropinized rat's colon when 1 ml. was added to the 15 ml. organ bath. The preparation of the colon was such that it contracted to 0.005 μg. or 0.01 μg. 5-HT. In one experiment the activity of 1 ml. perfusate corresponded to 0.01 μg. and in another to 0.02 μg. 5-HT. After the injection of 100 μg. of 48/80 the activity of 1 ml. effluent was sometimes equivalent to 0.6 μg.

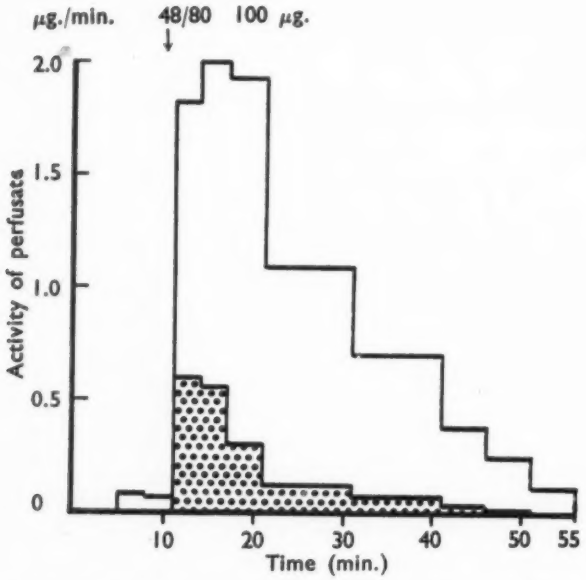

FIG. 3.—Comparison of the release of 5-HT and histamine by 100 μg. 48/80 injected intra-arterially to the perfused rat hindquarters. The open blocks represent histamine and shaded blocks represent 5-HT. The total outputs were 41 μg. histamine and 6 μg. 5-HT.

The time course of the liberation of 5-HT resembled that of histamine in that the maximum release occurred in the first samples collected after an injection of 48/80 and then declined during the following hour. This is shown in the experiment of Fig. 3, wherein the total amounts liberated were 6 μg. 5-HT and 41 μg. histamine. However, Fig. 4 shows that the release of 5-HT by 48/80 is more sudden than that of histamine. In the figure the percentage total output of both substances has been plotted against time. The results are from five experiments after the injection of 5 to 100 μg. 48/80. The scatter is large, but there is a significant difference between the values denoting 5-HT and those denoting histamine. Thus 5-HT appears to be released more suddenly than histamine since the rate of washing out from the site of release must be the same for both substances.

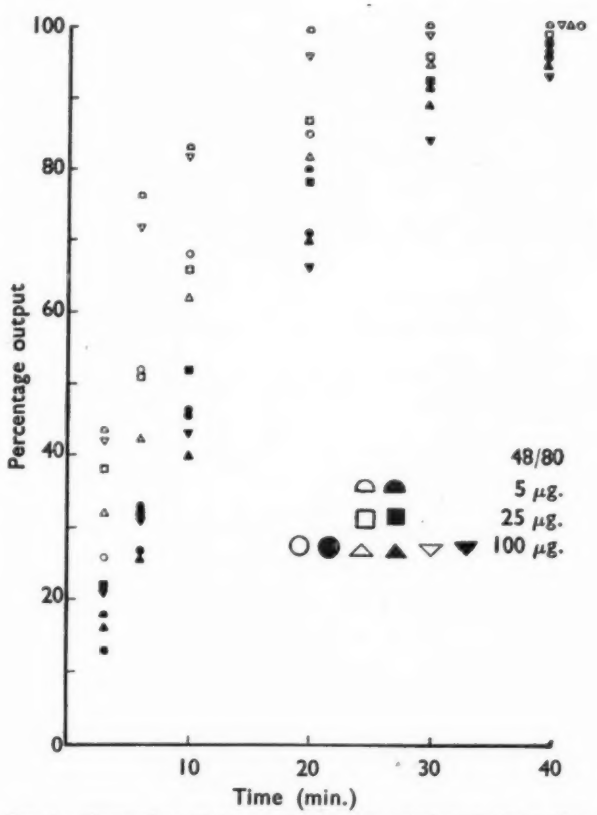

FIG. 4.—Comparison of the output of 5-HT and histamine from five perfused rat's hindquarters after injections of 5–100 μg. 48/80. The ordinates are percentages of total output of 5-HT and of histamine, the abscissae time in minutes. The open symbols (○ △ ▽ □ ⌓) represent 5-HT values and the closed symbols (● ▲ ▼ ■ ◓) histamine values.

Compound 48/80 released greater amounts of histamine than of 5-HT. This is shown in Table I. Usually the amount of 5-HT released was about 1/10 of that of histamine. With repeated injections of 48/80 the amounts of histamine released are

TABLE I

THE RELEASE OF HISTAMINE AND 5-HYDROXYTRYPTAMINE FROM THE PERFUSED HINDQUARTERS OF THE RAT

Compound Injected	Output of 5-HT (μg.)	Output of Histamine (μg.)
48/80:		
5 μg.	1·8	25
5 ,, ...	2·8	23
5 ,, ...	3·0	35
25 ,, ...	3·8	26
25 ,, ...	3·8	40
100 ,, ...	3·5	55
100 ,, ...	4·0	41
100 ,, ...	6·0	41
Propamidine, 5 mg.	5·0	38
Morphine sulphate, 1 mg. ..	2·8	1·5
5 ,, ..	8·1	2·2
10 ,, ..	10·2	3·4

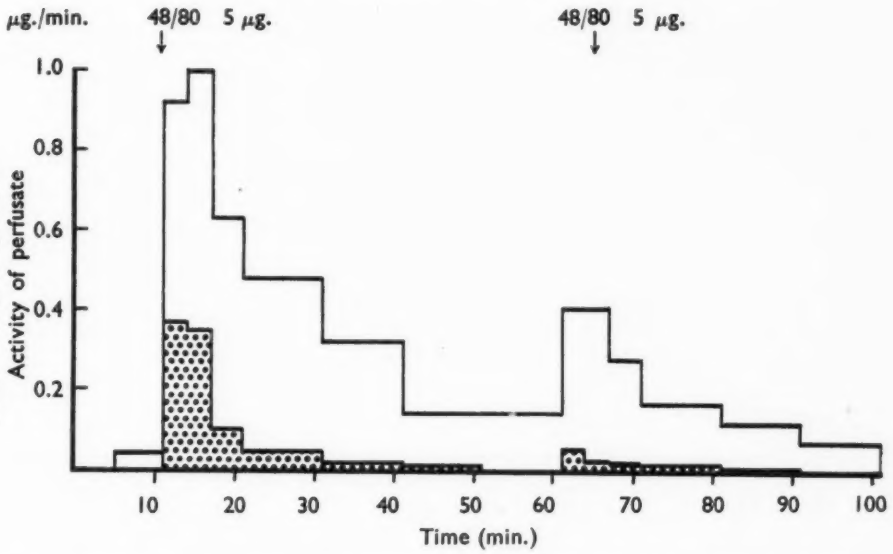

FIG. 5.—Comparison of the release of histamine and of 5-HT from the perfused rat's hindquarters after two arterial injections of 5 µg. 48/80. The open blocks represent histamine and the shaded blocks 5-HT. The total amounts liberated after the two injections were 23 µg. and 7·2 µg. of histamine and 2·8 µg. and 0·8 µg. of 5-HT.

known to decrease. This phenomenon also occurred in respect of 5-HT. In experiments in which 5 µg. of 48/80 was injected twice at an interval of 60 min. the amount of both histamine and of 5-HT released by the second injection of 48/80 was considerably less. Such an experiment is illustrated in Fig. 5. The amounts of histamine released after the two injections were 23 µg. and 7.2 µg.; the corresponding values for 5-HT were 2.8 µg. and 0.8 µg.

Other Histamine Liberators.—5-HT is released not only by 48/80 but also by propamidine and morphine. Propamidine acted like 48/80 in that the amounts of 5-HT released were smaller than those of histamine: with morphine the result was different, in each experiment the amount of 5-HT released was greater (see Table I).

Histamine.—Histamine caused no release of 5-HT; 25 µg. histamine was injected into the perfusion stream, and although the injected histamine was recovered in the venous effluent it did not contract the atropinized rat's colon and therefore did not contain 5-HT.

Rat Tissues from which 5-HT is Released

The technique of perfusion of the rat's hindquarters was such that the perfusion included the bladder, seminal vesicles and the testicles or the uterus. The 5-HT, however, did not originate from these urogenital organs, because, when both limbs were ligated as high as possible to minimize the amount of skin and muscle tissue being perfused, much smaller amounts of 5-HT, as well as histamine, were released by 100 µg. of 48/80, whereas removal of the urogenital organs before the injection of 48/80 did not reduce the release of either the 5-HT or the histamine from the perfused hindquarters. In addition, both substances were released from perfusions of single hind limbs.

A number of different experiments were performed to distinguish between skin and muscle as the source of the released 5-HT. The amounts of 5-HT released from the perfused isolated limb by 25 µg. 48/80 varied between 0.8 and 2.5 µg., and the amount of histamine between 15 and 25 µg. In one experiment in which the limb was skinned down to the ankle, but the foot—which could not be easily skinned—not tied off, the amounts of 5-HT and histamine released by 25 µg. 48/80 were of the same order as the amounts released from the unskinned limb—1.8 µg. 5-HT and 18 µg. histamine. However, when the foot was tied off from the skinned limb, in subsequent experiments, so as to obtain absolutely skin-free preparations, the amounts of 5-HT and histamine released were greatly reduced. Between 0.05 and 0.35 µg. of 5-HT was released in these experiments by 25 µg. 48/80; the amount of histamine released varied between 1 and 5 µg. These experiments show that not only is the skin the main source of the 5-HT released, but that a large proportion of the 5-HT comes from the skin of the feet.

In other experiments the gastrocnemius muscle and skin were perfused separately. The amount of 5-HT released from the perfused gastrocnemius muscle was only 0.005 µg./g. muscle, but from the perfused skin the amount calculated to be released was 0.1 µg./g. This value is probably too low, because, during the rigorous operative pro-

cedures necessary for isolating the skin, vascular damage rendered many small areas of skin inaccessible to the perfusion fluid so that in every experiment the estimated weight of tissue perfused was an overestimate. It is interesting that in these experiments the amount of histamine released from muscle, 0.75 μg./g., was almost as much as that released from skin, 0.8 μg./g.

Further evidence that the skin is the main source of the 5-HT was afforded by the fact that when a patch of skin was soaked *in vitro* in saline containing 48/80 both histamine and 5-HT were released. In one such experiment in which a patch of skin was soaked in a 1 in 1,000 48/80 solution at room temperature 2.3 μg. 5-HT and 13.3 μg. of histamine per gram tissue were released.

It is known that 48/80 releases histamine from the mast cells which abound in the skin and subcutaneous tissue, and it has been reported that the mast cells of the rat contain 5-HT as well (Benditt *et al.*, 1955). The following facts are in accord with the conclusion that the 5-HT released by 48/80 is derived from the mast cells:

(*a*) In the experiment in which the patch of skin had been soaked in 48/80 and had released large amounts of 5-HT and histamine, the underlying subcutaneous tissue when examined histologically showed that the majority of the mast cells were disrupted.

(*b*) The skin of the foot which is particularly rich in mast cells proved to be a main source of released 5-HT.

(*c*) Subcutaneous tissue which is also known to be rich in mast cells releases both histamine and 5-HT when incubated with 48/80. Table II shows the amounts of 5-HT and histamine released in these experiments during 30 min. incubation.

TABLE II

RELEASE OF 5-HYDROXYTRYPTAMINE AND HISTAMINE FROM SUBCUTANEOUS TISSUE INCUBATED WITH 48/80

48/80 (μg./ml.)	5-HT (μg./g.)	Hist. (μg./g.)
0	0·4	3
25	0·7	5
500	1·7	15

The results in Table II show that there is a relatively large spontaneous release of 5-HT and histamine from the subcutaneous tissue, probably as the result of injury during the dissection leading to disruption of some mast cells which could be seen with histological examination. The amounts released when subcutaneous tissue was incubated with 25 μg./ml. 48/80 may therefore have been due to such "spontaneous" release. The much larger amounts released by 500 μg./ml. 48/80, however, must be attributed to an action of this compound on the mast cells most of which, on histological examination, were found to be disrupted.

TABLE III

THE RELEASE OF HISTAMINE AND 5-HYDROXYTRYPTAMINE BY 48/80 FROM PERFUSED TISSUES OF CATS, DOGS, RABBITS AND RATS

Preparation	48/80 Injected (μg.)	Output of 5-HT (μg.)	Output of Histamine (μg.)
Cat hindquarters	100	0	30
	200	0	38
Cat skin	1	0	20
	5	0	26
Cat gastrocnemius muscle	25	0	6·5
Dog hind limb	500	0	10
Rabbit hindquarters	200	0	1
	500	0	0·5
Rat hindquarters	5	2·6	28
	25	3·8	33
	100	4·5	46
Rat hind limb	25	1·8	22

Perfusion of Rabbit's, Cat's and Dog's Tissues

Compound 48/80 releases histamine but no 5-HT from the perfused cat's, dog's and rabbit's tissues. This is shown in Table III. For comparison the mean amounts of 5-HT and histamine released from the perfused rat's tissues are included at the bottom of the table.

DISCUSSION

The present experiments show that, in rats, histamine is not the only smooth muscle stimulating substance released by histamine liberators, but that these liberate 5-HT as well. No evidence was obtained for the release of 5-HT by 48/80 from perfused tissues of dogs, cats or rabbits. It may therefore be that the release of 5-HT by histamine liberators is so small as not to be detected by the methods of assay used in the present experiments, or is specific for the rat, or at least not common for all laboratory species.

It is generally assumed, from the many observations which started with those of Riley and West (1952, 1953, 1955), that the mast cells are the main source of histamine in many, although not in all, tissues and that histamine liberators disrupt the mast cells and in doing so release their histamine (Riley, 1953). The recent report of Benditt *et al.* (1955) that the mast cells in the rat contain both histamine and 5-HT would suggest that the 5-HT released by histamine liberators in this species is also the result of disruption of the mast cells. The

present observation, that the skin and subcutaneous tissue, and particularly the skin of the feet, contribute to a large extent to the release of 5-HT after compound 48/80, is in accord with this conclusion, because mast cells abound in these rat tissues. If it should prove that the 5-HT and the histamine released by 48/80 and other histamine liberators originate solely from the mast cells it may be justified to use the term "mast-cell depleters" or perhaps even "mast-cell disruptors" instead of histamine liberators. The failure to obtain evidence for the release of 5-HT in cats, dogs and rabbits may be because the mast cells in these species do not contain 5-HT.

Paton (1951) has shown that in the cat 48/80 releases an unidentified slow contracting substance. No evidence for such a release was obtained in the present experiments on perfused cat's hindquarters, skin and muscles, but this does not exclude the possibility that the unidentified slow contracting substance obtained by Paton in the circulating blood of cats after intravenous injection of 48/80 was released from mast cells of other tissues in the body.

5-HT is apparently not a constituent of the mast cells of all species. Our results further indicate that in the rat the mast cells of various tissues contain histamine and 5-HT in different proportions. For instance, about the same amounts of histamine were released by 48/80 from the perfused gastrocnemius and skin of the rat—0.75 μg. and 0.8 μg./g. tissue—whereas the amounts of 5-HT released in the same experiments were 0.005 μg. and 0.1 μg./g. tissue. This suggests that the mast cells of skeletal muscle contain much less 5-HT in relation to histamine than the mast cells of the skin. Our results with morphine and 48/80 show that the proportion of histamine and 5-HT released from the same tissue by different histamine liberators varies. For instance, morphine released more 5-HT than histamine from the perfused hindquarters, whereas 48/80 released more histamine than 5-HT. Thus the two smooth muscle stimulating substances assumed to reside in the mast cells of rat tissue show a different sensitivity for their release by different histamine liberators or "mast cell depleters."

After 48/80 the maximal release of 5-HT occurred earlier than that of histamine. This suggests a somewhat different mechanism for the release of these two substances and raises the question whether the release of the one is dependent upon, or even influenced by, the release of the other.

The release of 5-HT cannot be accounted for by the action of the histamine released by injected 48/80; histamine did not liberate 5-HT. Feldberg and Smith (1953), on the other hand, found that large doses of tryptamine and 5-HT liberated histamine; they considered the possibility that the release of histamine might in some way be preceded by the release of 5-HT. In this connexion the present finding that the release of 5-HT preceded that of histamine is of interest. Nevertheless, it would be unjustified to assume from this fact alone that the release of histamine is dependent on that of 5-HT, since histamine is also released by 48/80 in those species in which there is no release of 5-HT.

It is known from the work of Jorpes (1946) that mast cells contain heparin and that in dogs their disruption releases heparin and renders the blood incoagulable. In rats the disruption of mast cells evoked by either anaphylactic or peptone shock or compound 48/80 does not change the blood coagulation time (Mota, Beraldo, and Junqueira, 1953). Riley, Shepherd, West, and Stroud (1955) have recently taken up this problem and have shown that in rats treated with 48/80 the tissues lose not only their histamine but their heparin as well, although in different proportions; a 95% loss of histamine was associated with a 53% loss of heparin, but this loss of heparin was not accompanied by any sign of its appearance in the circulating blood. It may well be, as suggested by Riley *et al.* (1955), that heparin in this species is concerned with events in the tissues rather than with the coagulability of the blood. The finding that histamine and heparin are not released to the same extent by 48/80 may be another instance in which two substances which reside in the mast cells of rat tissue show different sensitivity for their release by 48/80. As stated by Mota *et al.* (1953), the way histamine may be bound to the mast cell's cytoplasm and its relation to heparin constitutes a challenging problem.

No experiments have been performed to find out whether 48/80 depleted the rat's skin of its 5-HT as it depletes its histamine, nor do we know the exact amounts of 5-HT present in rat tissue. Feldberg and Smith (1953) stated, in their paper on the release of histamine by tryptamine and 5-HT, that acid saline extracts of rat's skin contracted the isolated rat's colon and that this activity corresponded to 10–20 μg. of tryptamine/g. tissue. It seems not unreasonable to assume that the unidentified rat colon-contracting substance of their skin extracts was 5-HT. Since 5-HT is about 50 times as active on the rat colon as tryptamine, the activity of the acid saline extracts of the rat's skin would correspond to 0.2 to 0.4 μg./g. tissue of

5-HT, amounts of the same order as those released by 48/80 from rat's skin.

Reserpine causes a reduction of 5-HT in the intestine and central nervous system (Brodie, Pletscher, and Shore, 1955; Pletscher, Shore, and Brodie, 1955). It is not known whether the 5-HT in these tissues resides in mast cells, the reserpine acting as a "mast cell depleter." The histamine in the intestinal mucosa is mainly resistant to histamine liberators and is apparently not of mast cell origin; if this also applies to the 5-HT in the intestinal wall and in the central nervous system, its release from these tissues by reserpine would be present an entirely different phenomenon.

Summary

1. The histamine liberators compound 48/80, propamidine, and morphine release from the perfused rat's tissues histamine and another smooth muscle stimulating substance which has been identified as 5-hydroxytryptamine (5-HT).

2. No evidence could be obtained for the release of 5-HT by 48/80 from the perfused tissues of cats, dogs and rabbits.

3. It is concluded that the 5-HT released by histamine liberators, like the released histamine and heparin, originates in the mast cells and is released from them.

4. It may become necessary in future to use a terminology which makes it clear that histamine liberators which act on the mast cells also release from them substances other than histamine. "Mast-cell depleters" is suggested as an appropriate term.

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DISTRIBUTION OF MEPACRINE IN THE ORGANS OF DIFFERENT ANIMAL SPECIES, AND IN THE COMPONENTS OF LIVER CELLS

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(RECEIVED FEBRUARY 16, 1956)

In a previous paper (Hurst, Snow, and Roberts, 1955) it was shown that the marked therapeutic effect of mepacrine dihydrochloride dihydrate (mepacrine) against equine encephalomyelitis in the mouse (Hurst, Melvin, and Peters, 1952; Hurst, Peters, and Melvin, 1952) could not be reproduced in a number of other animal species. The drug appeared to have no effect, or even an adverse effect, on infections in chicks, guinea-pigs, rabbits, and monkeys. Only in the adolescent rat could a significant protective action be demonstrated. Mepacrine failed to protect newly weaned rats from infection, whereas it had a good therapeutic action on similarly immature mice.

Since mepacrine is quite exceptional in showing a therapeutic action against some smaller viruses, it seemed important to try to discover why its action should be largely confined to the mouse. Various reasons for this selective action could be imagined. There might, for instance, be a difference in metabolism in the mouse leading to the production of an active substance not formed in other species. The importance of metabolism in the action of mepacrine is still an unsettled question. On the one hand there is some biological evidence favouring the possibility that mepacrine may exhibit its antiviral activity because of conversion *in vivo* into an active product; on the other hand there is the observation (Goodall, 1956) that the acridines extractable from livers of mepacrine-treated mice contain at least 96% of unchanged mepacrine either free or in the form of salts with phospholipids. Another possible way in which the mouse could differ from other species is in the mode of distribution of mepacrine, either in the different tissues of the body or in different cellular components. It is this possibility which has been examined in the experiments described here. Although scattered references to the concentrations of mepacrine in various animal tissues occur in the literature, it has not been possible to make use of them. Most of them, e.g. Hecht (1936), Oldham and Kelsey (1945), Dearborn (1947), refer to small single doses of mepacrine or to small amounts administered daily over a period, whereas others give results which are insufficient for any comparisons to be made (e.g. Scudi and Hamlin, 1943). A systematic comparison of the levels of mepacrine in organs of five different animal species has therefore been made at various times after oral administration of a single massive dose of the drug.

Another line of approach was suggested by the observation (Hurst *et al.*, 1955) that liver cells of mice treated with mepacrine contain yellow cytoplasmic particles which fluoresce and which may be stained with basic dyes; certain other species such as the guinea-pig do not exhibit this effect. It was thought that the quantitative distribution of mepacrine in the different component parts of the liver cell might be important in relation to the antiviral action of the drug. Experiments involving the centrifugal separation of cellular particles were therefore performed with livers from mice and guinea-pigs previously treated with mepacrine.

METHODS

Animals

The animals used and their weight ranges were as follows (dose of mepacrine in mg./kg. in parentheses): mouse, 20–25 g. (500); rat, 230–300 g. (500); guinea-pig, 200–250 g. (200); rabbit, about 2,000 g. (300); chick, 40–50 g. (400). Mepacrine was given as a single oral dose, guinea-pigs being dosed with a fine catheter. Animals were decapitated or stunned and exsanguinated; organs for mepacrine determination were stored at −10°; livers for separation of cell components were homogenized at once, or, where the period after treatment was 6 or 12 hr., were stored overnight at 2°.

Determination of Mepacrine in Tissues

The weighed tissue was homogenized in water (10 ml./g. tissue) in a Waring blender. While the machine was

still running quickly enough to keep the suspension homogeneous a portion (1 or 2 ml.) was removed and shaken for 30 sec. with butanol (4 ml.). After centrifugation the solvent layer was removed and the aqueous layer and tissue extracted further with butanol (2 ml./extraction). A total of three butanol extractions usually sufficed, but occasionally a fourth was needed. The combined butanol extracts were mixed with ether (20 ml.) and extracted twice with 0.1 N-HCl (5 ml. and 3 ml.). The combined acid extract was diluted to 10 ml. and the optical density measured at 430 mμ on a Beckman spectrophotometer (1–4 cm. cell according to density). The result was converted to μg. of the mepacrine salt/g. moist tissue taking the extinction value 9,100 for the mepacrine absorption.

The procedure gave a quantitative extraction for all tissues except chick liver, where the yellow acridine-like material in the butanol extract could not be completely extracted into hydrochloric acid. The numbers given in the table refer only to acid-extractable material. The non-extractable product, which was present in concentration comparable to that of the mepacrine, gave an absorption spectrum in *iso*propanol differing widely from that of mepacrine, and showing maxima at 452 and 470 mμ.

Separation of Cell Components and Determination of Acridine Contents

The homogenization of the livers and separation of cell components were carried out at 0–5° with sucrose solution as the suspending medium. For convenience the tissue pulp was divided; one part was used for separation of nuclei and the other for the smaller cell components. Details are given in Table I.

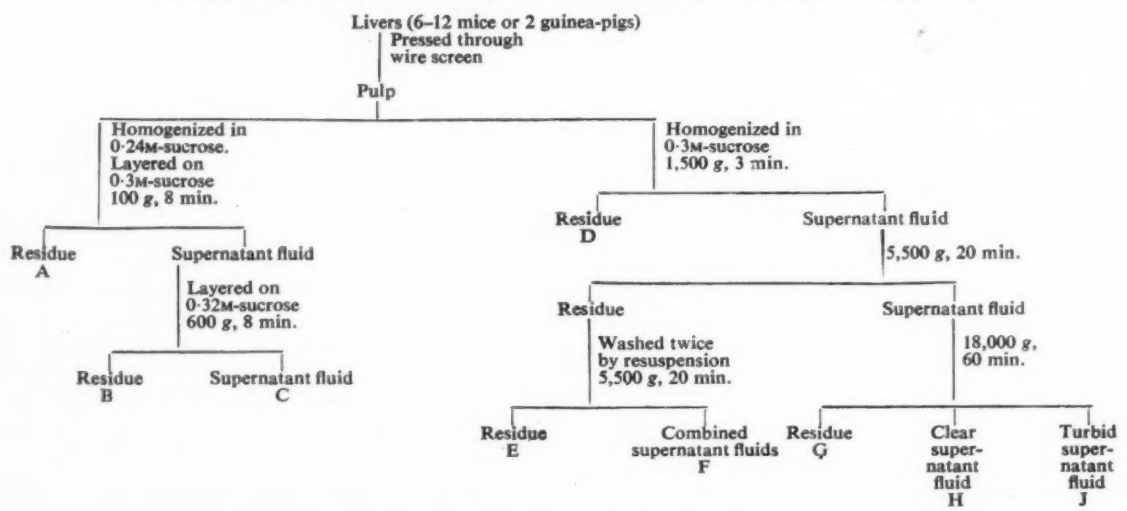
Total acridine was determined in all component suspensions (except J) by the method used for tissues. A check on recovery was made by totalling the contents estimated for fractions A–C and those for fractions D–H. A difference of not more than 10% between these totals was regarded as tolerable having regard to the possible summation of errors on a large number of estimations and the inevitable mechanical losses in the operations. The known loss in fraction J was usually negligible. The calculation of the actual acridine content of the different cellular components from the measured contents of the fractions separated experimentally required some consideration of the cellular composition of these fractions. It was possible to arrive at fairly reliable figures by utilizing the fact that the concentration in the mitochondrial particles was always very much higher than in the microsomal particles or the cell plasma. In discussing the method of calculation, the different cellular components will be considered in turn.

Nuclear Component.—Fraction A, which was nominally cellular debris, contained considerable quantities of nuclei. Fraction B contained little but nuclei whereas there were only occasional nuclei to be seen in fraction C. The acridine content of fraction A and B was usually of the same order. Since there were so many nuclei in A the nuclear component was taken as the sum of A+B. The mepacrine values for the nuclear component are thus rather too high, since they include any acridine contained in connective tissues and in a few unbroken cells.

Mitochondrial Component.—The mitochondrial particles were present in fractions D, E, and F. In D were found the largest of the mitochondrial particles accompanied by the whole of the nuclei and debris; E contained little but mitochondrial particles whereas F contained mitochondrial particles with perhaps 15–20% of the total cell plasma and microsomes. The latter made

TABLE I

SEPARATION OF CELL COMPONENTS FROM LIVERS OF MOUSE OR GUINEA-PIG



Figures indicate centrifugal acceleration and time.
* Part of supernatant fluid is always turbid because of unavoidable disturbance of the residue in removal of the last drops.

only a small contribution to the acridine content and the slight excess due to this source was left uncorrected to offset partially the unavoidable mechanical losses. The total acridine in the mitochondrial fraction was therefore calculated as D+E+F−(A+B). (Sum of acridine in all mitochondria-containing fractions less the acridine known to be present in the debris and nuclei.)

Microsomal Component.—Consideration of the volumes of extracts and precipitates at various stages of the separation showed that the microsomal component must be widely distributed in different fractions and fraction G can only represent a part of the total. Microsomes are lost in fractions D, F, and J. It was estimated that recovery of microsomes in G was of the order of 50%. For the purposes of these calculations therefore, the mepacrine content of fraction G has been doubled to give a figure for the content of the microsomal fraction. This is admittedly only a crude approximation and the mepacrine values for this component must be regarded as rather inaccurate.

Cell Plasma.—A fairly exact determination of the content of cell plasma could be made. Since the original preparation involved the homogenization of liver with sucrose solution in the ratio 5 ml. sucrose/g. liver, the volume of plasma+sucrose solution in the suspension could reasonably be taken as 5.32 ml./g. liver. The final clear supernatant fluid (H) after removal of all the particulate cell components could be regarded as an aliquot of this solution and hence the acridine content of cell plasma determined.

Effect of Time of Dosing on the Therapeutic Response to Mepacrine in the Mouse and Rat

Newly weaned mice or young rats (30–35 g.) in groups of 20 were used. They received a single dose of mepacrine hydrochloride (350 mg./kg.) at times varying from 5 days before to 1 day after intramuscular infection with Eastern equine encephalomyelitis. Virus strains and conditions of experiment were as in previous investigations (see Hurst *et al.*, 1952). Mortalities and times of survival are given in Table III.

Results

Mepacrine Levels in the Organs of Different Species

The species chosen for comparison were mouse, rat, guinea-pig, rabbit, and chick. In order to make the experiments as nearly comparable as possible the animals were all given a single large oral dose. The amount chosen was the largest that could be given without causing evident toxic effects. Hence the doses were not identical on a weight basis, but varied by a factor of 2.5 from the highest (rat and mouse) to the lowest (guinea-pig). Various methods have been described for the extraction of mepacrine from tissues. In the older methods (Hecht, 1936; Dearborn, Kelsey, Oldham, and Geiling, 1943) the tissue proteins were denatured by heating with alkali or acid, but more recently the direct extraction method of Brodie and Udenfriend (1943) has been favoured. This involves the use of ethylene dichloride. In the present experiments butanol has been used as the extracting solvent. Ether was added to the butanolic extract and the acridine transferred to aqueous solution as hydrochloride; the optical density was measured at 430 mμ, which is near a peak in the mepacrine absorption spectrum. The results were calculated as mepacrine but represent the total acridine extractable under these conditions (assuming a comparable absorption at 430 mμ). In mouse liver it has been shown (Goodall, 1956) that at least 96% of the extractable acridine is mepacrine. The composition in other tissues is unknown, but absorption measurements above and below 430 mμ showed that there was a true peak at this wavelength, which is probably characteristic of the acridine nucleus present in mepacrine. Metabolic change in the side chain would not be revealed as a change of spectrum unless the whole side chain were lost. The only anomaly in this extraction procedure was found with chick liver, where it was possible to extract only a part of the acridine from the organic solvent phase into 0.1 N-HCl. Examination of the yellow material remaining in the organic solvent layer showed that it was not a mepacrine complex with phospholipid. It could not be extracted into acid solution after shaking with sodium hydroxide solution, and its absorption spectrum was markedly different from that of mepacrine. It is therefore evident that chick liver accumulates a metabolite of mepacrine in amounts comparable with the mepacrine present; results are given only for the basic material behaving as mepacrine. Acridine determinations were carried out at various times after treatment with mepacrine; the results are set out in Table II, which also shows the number of animals contributing to each result. The figures are mostly mean values of 2–6 determinations on smaller sub-groups of animals. Where marked discrepancies were found the determinations were repeated.

The figures show certain distinctive differences in the distribution of acridine in the tissues. Mouse and rat were outstanding in showing liver concentrations rising gradually to a high peak. In mouse liver the peak was about twice the 6-hour level and was reached in about 24 hours, but in rat liver the peak was five times the 6-hour level and was reached later at about 4 days after treatment. By contrast, in the other species the maximum was almost reached in six hours. In guinea-pig and chick this concentration began to fall after 24 hours, but in the rabbit the rather low maximum was maintained with

TABLE II

TOTAL ACRIDINE IN ORGANS OF DIFFERENT SPECIES AFTER A SINGLE ORAL DOSE OF MEPACRINE

For details of dose see text. Total acridine as μg. mepacrine salt/g. moist tissue. Figures in parentheses show number of animals contributing to each mean value.

Organ	Species	Content at Given Time after Treatment							
		3 hr.	6 hr.	12 hr.	24 hr.	2 days	4 days	8 days	16 days
Liver	Mouse	950 (12)	1,350 (24)	1,840 (42)	2,330 (24)	1,560 (18)	1,330 (12)		80 (12)
	Rat		520 (2)	680 (2)	1,630 (2)	2,300 (6)	2,520 (2)	2,550 (6)	650 (2)
	Rabbit		430 (4)	430 (5)	480 (2)	610 (5)	560 (2)	60 (6)	70 (5)
	Guinea-pig		640 (4)	710 (3)	810 (4)	640 (7)	120 (4)		
	Chick*		680(120)	530(120)	880 (60)	700 (30)	300 (30)		8 (30)
Spleen	Mouse	480 (12)	350 (42)	770 (24)	450 (24)	390 (18)	120 (12)		30 (12)
	Rat		160 (2)	190 (2)	330 (2)		640 (8)	410 (6)	390 (2)
	Rabbit		610 (4)	470 (5)	500 (2)	400 (5)		60 (3)	30 (2)
	Guinea-pig		420 (4)	310 (3)	700 (4)	610 (3)	130 (4)		
	Chick		650 (60)	1,080 (90)	1,040 (30)	1,210 (30)	390 (30)		0 (30)
Kidney	Mouse	420 (12)	520 (42)	690 (42)	450 (24)	370 (18)	170 (12)		50 (12)
	Rat		120 (2)	90 (2)	200 (2)	400 (6)	450 (8)	420 (6)	310 (2)
	Rabbit		570 (4)	420 (5)	390 (2)	620 (5)	740 (2)	180 (3)	110 (2)
	Guinea-pig		170 (4)	270 (3)	370 (4)	220 (3)	150 (4)		
	Chick		180 (60)	190 (90)	250 (30)	100 (30)	50 (30)		1 (30)
Brain	Mouse		16 (12)	28 (12)	16 (12)		20 (12)		12 (12)
	Rat		7 (2)	5 (2)	8 (2)		14 (2)		7 (2)

* Results for chick liver refer only to acridine extractable into acid solution.

little change for at least 4 days. The acridine concentrations in the spleen, compared with the kidney of the same species at any particular interval after treatment, were closely similar in the mouse, rat, and rabbit. In the guinea-pig and chick the spleen levels were much higher than those in the kidney, but changes in the two organs at different times after treatment were roughly parallel. For the rat and mouse the maximum spleen values were a small fraction of those in the liver, whereas in the other species the level in the spleen approached or even exceeded that in the liver. Acridine concentrations in spleen and kidney usually showed a relatively small rise after 6 hours to a maximum, followed by a slow decline. The maximum occurred in mice and chicks at 12–24 hours, in guinea-pigs at 24–48 hours, and in rats and rabbits at about 4 days. The persistence of acridines in the rat was most marked. Substantial amounts remained in liver, spleen, and kidney 16 days after treatment, whereas in all other species the values had dropped markedly. A similar observation with rats was made by Scudi and Hamlin (1943).

Considering these results, it appeared that the only characteristic which distinguished the mouse from all the other species in the distribution of acridines was the rapid development of very high concentrations in the liver. Equally high levels were eventually reached in the rat, but only after a delay of several days. If the requirement for antiviral activity were the presence of a high concentration of acridine in the liver within a few hours of virus inoculation, it might be expected that mepacrine would show a good antiviral effect in the rat, provided that infection were postponed until 3–4 days after treatment with the drug. Experiments to test this possibility were therefore carried out.

The Effect of Mepacrine in the Mouse and Rat According to the Time of Administration Relative to That of Infection

In the mouse we have consistently obtained the maximal therapeutic effect of mepacrine when the drug has been given at the time of, or 24 or 48 hours before, infection with Eastern equine encephalomyelitis. In older animals reasonable, or sometimes quite striking, effects have followed administration 4 or 24 hours after virus, but, even in these, prior treatment with mepacrine has yielded better results. This suggests that it is necessary for a period to elapse, either to enable some metabolite to be formed or for mepacrine itself to become closely associated with some critical cellular component, before extraneural infection is fully established if the maximal therapeutic response is to be obtained. If the antiviral effect were directly related to the concentration of drug in the liver, it might be expected that the temporal relations would be altered as mentioned in the previous paragraph. The data presented in Table III give no indication of this. With the very young (and more highly susceptible) mice used, the therapeutic effect was less marked than in older animals, but it is clear that the optimal period for administration of drug is as stated above, and that in this respect no difference exists between the mouse and the rat. However, the fact remains that the concentration of mepacrine in the liver of mouse and rat is out-

TABLE III

EFFECT OF TIME OF TREATMENT ON THE THERAPEUTIC RESPONSE TO MEPACRINE IN THE MOUSE AND RAT

Figures show number of deaths in groups of 20, and mean periods (days) of survival in parentheses.

Time of Treatment	Expt. 1		Expt. 2	
	Mice	Rats	Mice	Rats
No drug	20 (4·6)	5 (7·0)	20 (5·3)	10 (6·1)
5 days before virus	16 (7·0)	4 (6·8)	14 (6·1)	5 (5·2)
2 „ „ „	9 (7·6)	1 (8·0)	9 (7·8)	3 (6·6)
1 day „ „	7 (8·1)	0	6 (8·2)	1 (7·0)
1 „ after „	19 (4·2)	3 (8·7)	17 (4·5)	2 (5·5)

standingly higher than in species insusceptible to chemotherapy with the drug.

Separation of Cell Components and Determination of Mepacrine Contents

The method of separation used was essentially that of Hogeboom, Schneider, and Pallade (1948) with some modifications. The preparations were examined microscopically as fixed and stained smears on slides. The fractionation was designed to yield fairly pure preparations of nuclear, mitochondrial and microsomal particles together with intermediate fractions containing mixtures of particles. Any separation of this kind must be a compromise between high yield and high purity of any particular cell component. Consequently it is difficult to estimate the total content of a drug in any particular component. In these experiments the high mepacrine content of the mitochondrial fraction in relation to other fractions made the task somewhat simpler, since small amounts of microsomes and cell plasma contaminating the mitochondrial fraction would contribute little to the total acridine present. The results are shown in Table IV. It will be seen that in mouse liver, after a single large dose of mepacrine, the acridine tends to be concentrated mainly in the mitochondrial particles, and to a less extent in the nuclei, with relatively low concentrations in the microsomal particles and the cell plasma. The ratio of acridine present in the mitochondrial particles to that in the nuclei rises from about 2 at 3 to 6 hours after treatment to 4 at 2 to 4 days and then declines again. At the peak of acridine concentration in the mouse liver 60–70% is found in the mitochondrial particles. Figures for the cell components of the guinea-pig are less extensive, but serve to show that in this species also the amounts of acridine in the mitochondrial particles are greater than in any of the other cell components. The acridine ratio (mitochondrial component/nuclei) varies from 3 at 6 hours after treatment to less than 2 at 24 and 96 hours.

The results of the distribution of acridine in mouse liver components are in direct contrast to those of Tomkins and Brodie (1954) for rat livers. Brodie claims that mepacrine is very largely concentrated in the nuclei where it is firmly held. A single experiment, under our conditions, with rats suggested that in rats as in mice and guinea-pigs the main concentration was in the mitochondrial particles.

TABLE IV

TOTAL ACRIDINES IN CELL COMPONENTS OF MOUSE AND GUINEA-PIG LIVER AT VARYING TIMES AFTER A SINGLE DOSE OF MEPACRINE

Mean total acridine as μg. mepacrine salt/g. liver.

Separated Fractions	Animal:	Mouse								Guinea-pig		
	Time after dose (hr.):	3	6	12	24	48	96	190	380	6	24	190
	No. of separate expts.:	2	3	2	2	2	3	2	1	2	1	1
A		120	140	190	330	310	200	140	7	55	120	9
B		160	220	230	210	210	160	150	7	64	76	12
C		720	960	1,300	2,120	2,150	1,340	580	40	520	520	84
D		590	700	930	1,220	1,360	1,140	450	30	330	340	22
E		170	280	520	750	690	450	340	20	65	160	21
F		80	70	200	370	320	150	50	0	77	64	14
G		80	80	110	110	190	60	20	4	74	53	6
H		80	120	70	150	240	90	30	4	85	65	28
Calculated Content of Components												
Nuclear		280	360	420	540	520	360	290	14	120	200	21
Mitochondrial ..		560	690	1,230	1,800	1,850	1,380	540	36	360	370	36
Microsomal ..		160	160	220	220	380	120	40	8	150	110	6
Cell plasma ..		80	120	70	150	240	90	30	4	85	65	28
Total		1,080	1,330	1,940	2,710	2,990	1,950	900	60	715	745	91
Acridine ratio: mitrochondrial/ nuclear fractions		2·0	1·9	2·9	3·3	3·6	3·8	1·9	2·6	3·0	1·9	1·7

It seems likely that the distribution depends on the dose administered. Brodie's conditions (private communication) were daily intraperitoneal doses of mepacrine at 50 mg./kg. He was later able to confirm our results with large oral doses.

Discussion

Although the pattern of distribution of mepacrine in different organs varies considerably from one species to another, the only feature which is clearly characteristic of the mouse is the rapid establishment of high levels of acridine in the liver; in the rat similar levels are reached, but only after some delay. The evidence presented suggests that, although there may be a correlation between the high mepacrine levels in the livers of these two species and the antiviral action of the drug, there are certainly other unknown factors involved.

The distribution of drugs within the cell itself has rarely been studied. The present investigation has provided an example of the possibility of doing so, and of some of the precautions required in interpreting the results. It has also shown that mepacrine can be largely concentrated in a particular cell component—under the conditions of these experiments in the mitochondrial fraction—and that the relative concentrations in different parts of the cell may change at different times after treatment.

The possible correlation between the local concentration of mepacrine within the liver cell and its antiviral effect can only be considered in relation to the distribution of virus within the cell. This has been the subject of further experiments which will be discussed in a later publication.

Summary

1. Concentrations of mepacrine in liver, spleen, and kidney resulting from a single massive dose have been measured in mice, rats, guinea-pigs, chicks, and rabbits at different times after administration.

2. Mice and rats—the only species in which mepacrine exhibits a protective action against infection with Eastern equine encephalomyelitis—are distinguished by development of exceptionally high concentrations in the liver. Although this correlation may be significant, evidence suggests that other factors must play a part in determining the antiviral action of mepacrine.

3. With the dose of mepacrine used, the bulk of the acridine in the mouse liver was in the mitochondrial fraction, with decreasing amounts in the nuclear, microsomal and cell plasm fractions respectively. The difference in concentrations between the components was greatest when the total acridine in the organ was at a maximum. Distribution of the drug in the components of guinea-pig liver was probably similar.

The authors wish to thank Messrs. J. H. Sherwood and J. C. B. Smith for technical assistance in analysis.

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MEPACRINE METABOLISM: AN EXAMINATION OF MOUSE LIVER FOR POSSIBLE ANTIVIRAL METABOLITES

BY

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(RECEIVED FEBRUARY 24, 1956)

Hitherto, in the attempted chemotherapy of the smaller viruses, a few polymeric substances have shown slight activity, but compounds of lower molecular weight have not been effective. Recently, however, Hurst, Melvin, and Peters (1952) showed that mepacrine had a very considerable prophylactic effect on Eastern equine encephalomyelitis in the mouse, but not in the rabbit, guinea-pig, chick, or monkey, and only slightly in the rat. Thus it would appear that either the course of the infection in the mouse is not comparable with that in the other species, or the metabolism or distribution of mepacrine in the mouse is different, and that possibly a very active antiviral metabolite is formed. The latter hypothesis forms the reason for the present work. Staining of mouse liver, spleen, and skin persists for several days after one oral dose. The liver was selected as a convenient organ in which to search for metabolic products, since relatively large amounts of acridine and virus accumulate in it ; furthermore, it is the site at which abundant basiphil particles are found in the cell cytoplasm (Hurst, Snow, and Roberts, 1955).

METHODS

Adsorbents for Chromatography.—Alumina—Grade "0" manufactured by Peter Spence Ltd., Widnes. *Decalso*—Sodium aluminium silicate, manufactured by the Permutit Company, London.

Paper Chromatography of Acridines.—Whatman No. 50 filter paper sheet was immersed in an aqueous 0.1M buffer solution (*p*H 7.3, containing, per litre, potassium dihydrogen phosphate 13.6 g., sodium hydroxide 2.97 g., and trimethylcetyl-ammonium bromide, "Cetavlon," 1.0 g.). The paper was hung up and, when just damp, the solution of the sample was applied in the usual way. The paper sheet was transferred to a chromatographic tank containing filter paper curtains soaked in buffer solution and in ethyl acetate respectively. After the test sheet had been enclosed for 4 hr., 60 ml. of ethyl acetate saturated with the aqueous buffer solution was introduced into the trough, and the development was allowed to proceed during 12 hr. The solvent was allowed to drip off the end of the paper, the edges of which were serrated.

Extraction of Acridines from Liver

To remove mepacrine and any possible metabolites from mouse livers (taken 48 hr. after oral dosing at 500 mg./kg.) with a minimum of fat, protein, etc., the livers (300–400 g.) were disintegrated in a suspension of methanol or *iso*propanol (2 l.) using a Waring blender under a blanket of nitrogen. The suspension filtered readily, and after two further treatments each with 1 l. of the alcohol, only a very small proportion of the mepacrine remained adsorbed on the minced tissue. The combined alcohol extracts were stored in the refrigerator overnight, then filtered. The clear yellow filtrate was evaporated under reduced pressure in nitrogen to a volume of 1 l. This concentrate, on shaking with 1 l. of light petroleum (b.p. 60–80°), formed two distinct yellow layers having a green fluorescence. None of the yellow colour could be extracted from the petroleum solution on shaking with dilute hydrochloric acid. It was estimated colorimetrically that about 38% of the total acridine extracted was retained in the petroleum layer. The analytical work was thus divided into an examination of the aqueous and the petroleum layers.

Countercurrent Distribution of the Aqueous Layer.—The aqueous layer obtained after treatment of 890 g. of the livers as described above was brought to *p*H 6 with phosphoric acid and the volume adjusted to 50 ml. This solution was then subjected to six stages of countercurrent extraction between chloroform (500 ml.) and 0.1M-phosphate buffer (500 ml., *p*H 6). The colorimetric analysis of each stage indicated that there was a partial separation into two fractions in stages 1 and 2 and 3–6 inclusive. The manner in which the stages were combined and worked up is as shown in Table I. For the isolation of the 5–10% of unknown compound from the 480 mg. of impure mepacrine hydrochloride (stages 3–5, Tables I and III), samples were subjected to countercurrent distribution in two different systems: (i) chloroform and 0.1M-citrate buffer (*p*H 5.9), and (ii) ethyl acetate 0.1M-phosphate buffer (*p*H 7.3). The

TABLE I

COUNTERCURRENT EXTRACTION OF WATER-SOLUBLE MEPACRINE METABOLITES

Stage No.	Absorption* Density	Method of Isolation
1	0·7	The aqueous layer brought to *p*H 8, extracted with the chloroform layer, chloroform removed and residue triturated with ether; 21 mg. insoluble solids remained. From the ethereal filtrate 144 mg. of hydrochloride was precipitated.†
2	0·35	
3	0·8	Treatment as above led to an ether soluble product from which 480 mg. of hydrochloride was isolated.
4	0·8	
5	0·4	
6	0·08	The chloroform layer separated and extracted with *p*H 4 citrate buffer. Citrate extract brought to *p*H 8 and transferred to chloroform. After removing chloroform, the solid residue (7 mg.) was insoluble in ether.

* $d_{1\ cm.}$ at 435 mμ.

† The precipitation of hydrochloride is described in Table II, footnote.

results from (i) illustrated in Fig. 1A show a minor component in stages 38–46, with the major component spread over stages 15–35. The dotted line is a theoretical curve for a compound having p=0.59 (where p=fraction of the total distributed in the top layer). The slight divergence of the observed curve from the theoretical is probably due to experimental imperfections, yet the results are such that 5–10% of an impurity having p=0.66 could not be detected.

By using the higher order of separation obtainable from paper chromatography in conjunction with a higher *p*H and ethyl acetate as mobile phase, the sample gave rise to two spots. These were from a secondary component and mepacrine, together with a minute quantity of a third component made visible by ultraviolet irradiation (secondary and tertiary spots travelled 0.25 and 0.17 of the distance traversed by the mepacrine spot; the solvent front was beyond the end of the paper). The proportion of the secondary component was conveniently estimated by bulk countercurrent distribution as in Fig. 1B. Note that the positions of the main and secondary components are reversed in changing from systems (i) to (ii). The amount of the secondary component in stages 2–7 estimated by spectrophotometry at 425 mμ after dilution with 0.1N-hydrochloric acid was approximately 5% of the total fraction.

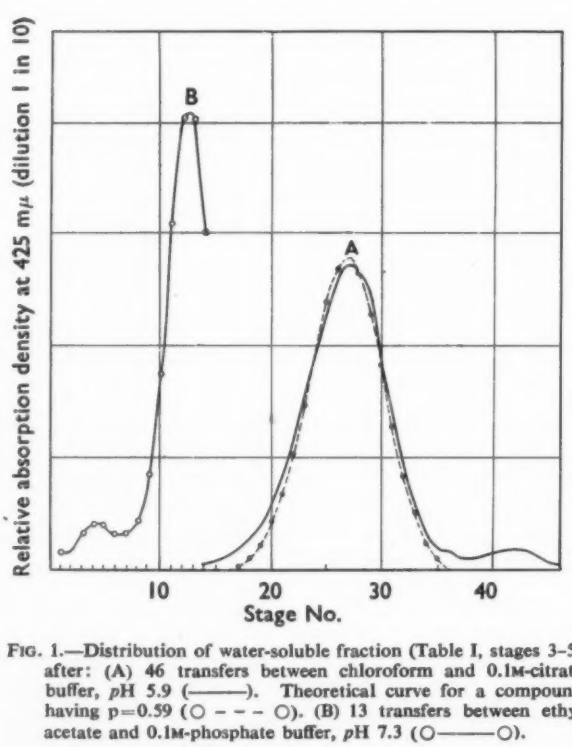

FIG. 1.—Distribution of water-soluble fraction (Table I, stages 3–5) after: (A) 46 transfers between chloroform and 0.1M-citrate buffer, *p*H 5.9 (———). Theoretical curve for a compound having p=0.59 (○ - - - ○). (B) 13 transfers between ethyl acetate and 0.1M-phosphate buffer, *p*H 7.3 (○———○).

In order to obtain milligram amounts of the secondary component, a fraction comparable to that from stages 3–5 above, prepared from 1,500 g. of livers, was subjected to 19 transfers in the ethyl acetate *p*H 7.3 buffer system. The secondary component separated in stages 1–10 was extracted into chloroform and the solution concentrated under reduced pressure in nitrogen. Then 10 volumes of *n*-hexane were added and after standing overnight at 5° a yellow amorphous precipitate was obtained and isolated. The behaviour on the paper chromatogram was identical with that of the secondary component already described. The precipitated base was soluble in water but insoluble in diethyl-ether.

Chromatography of the Petroleum Layer.—Whereas mepacrine base was strongly adsorbed from petroleum on to alumina, the petroleum extract from liver was slowly developed into 3 green fluorescent bands and one leading blue fluorescent band. A separation into three or four bands was even more readily effected on a decalso column, which was preferable on account of the lower alkalinity of the sorbent. After preliminary trials on the small scale, 250 g. of decalso (sieved to 120–200 B.S.S.) was suspended in light petroleum, and poured into a glass tube to make a column of adsorbent, 2½ in. ×5 in. Into this was poured 750 ml. of petroleum fraction representing the extract from approximately 1,200 g. of mouse liver (1,100 mice). Development was continued with light petroleum (b.p. 60–80°). The fractions and their analyses are detailed in Tables II and III.

Determination of "Mepacrine Binding Power."—Two ml. of an *n*-hexane solution of the acid to be tested (containing about 0.05 m-equiv.) was placed in a 10 ml. B.14 glass-stoppered tube containing 2 ml. of aqueous 0.1M-phosphate buffer solution (*p*H 6). The contents of the tube were titrated with mepacrine base solution in *n*-hexane (1 mg./ml.) added dropwise with shaking after each addition. The end-point was reached at the first appearance of a yellow colour in the aqueous layer. The "mepacrine binding power" was defined as that amount of the sample which retained 10 mg. of mepacrine base in the petroleum layer.

Isolation of Acidic Phosphatide from Mouse Liver.—Livers (727 g.) from untreated mice were disintegrated in 1 l. of *iso*propanol. The treatment of the solids

TABLE II

CHROMATOGRAPHIC FRACTIONATION OF PETROLEUM EXTRACT FROM 1,200 G. OF LIVERS FROM TREATED MICE

Fraction No.	Treatment on Decalso Column and Subsequent Isolation
1	A brown band with purple fluorescence was eluted with light petroleum (b.p. 60–80°) and separated after extraction with 0·1 N-HCl into: (a) 83 mg. total solids from the acid layer and (b) 2·9 g. of yellow oil having a purple fluorescence after evaporation of the petroleum.
2	A yellow band with green fluorescence was eluted with light petroleum and extracted with *p*H 6 aqueous buffer: (a) 86 mg. hydrochloride was isolated from the aqueous layer. (b) 4·6 g. of oil was isolated from the petroleum layer. The oil was washed with methanol and chilled, and 0·202 g. of white crystals (m.p. 147° C.) remained.
3	A yellow band was eluted with 2 l. of petroleum ether and extracted with *p*H 6 aqueous buffer solution. (a) 106 mg. hydrochloride was isolated from the aqueous layer.* (b) 1·24 g. of oil remained after removing the petroleum.
4	A brown-yellow band was eluted with acetone, transferred to light petroleum and separated after extraction with *p*H 2 aqueous buffer into: (a) a yellow oil obtained from the aqueous layer after basification and extraction with chloroform. The oil did not yield a hydrochloride. (b) 0·86 g. of gum from the petroleum layer.
5	A fixed yellow band was excised from the top of the column and extracted with dilute hydrochloric acid: 26 mg. of hydrochloride was isolated from the aqueous acid extract.

* The hydrochloride was obtained from the aqueous solution after alkalinization, extraction into chloroform, transfer to dry diethyl-ether, and precipitation by passage of dry hydrogen chloride. The precipitated hydrochloride was washed with ether till free from acid.

obtained after spinning was repeated with two further 1 l. portions of *iso*propanol. The combined supernatant liquids were evaporated under reduced pressure and the concentrated aqueous-alcoholic layer extracted with light petroleum (b.p. 60–80°). The aqueous layer was rejected. To separate acidic phosphatide from neutral constituents the petroleum layer was extracted with 4N-sodium hydroxide. The extracted petroleum layer was rejected and the alkaline layer acidified whilst cooling, and extracted with two successive portions of *n*-hexane. The combined hexane layers were washed with water and evaporated to 25 ml.; 2.9 g. of total solids were in the hexane. The solids contained phosphorus and nitrogen but no sulphur, and had a mepacrine binding power of the same order (240 mg. binds 10 mg. of mepacrine) as that obtained from the treated liver (cf. Table II, fraction 2(b), after washing with acid).

Mepacrine Salt of Inositol Phosphatide.—Two g. of the inositol phosphatide fraction from ox-brain (Folch, 1942) was dissolved in 25 ml. of light petroleum (b.p. 40–60°), shaken with 25 ml. 2N-HCl and washed with water. After the addition of 200 mg. mepacrine base in 15 ml. of light petroleum, the colour changed to deep orange, indicating the formation of a salt. Water (40 ml.) was added and the petrol was removed *in vacuo*. Mepacrine phosphatide was obtained as a soft flocculated yellow precipitate. It was insoluble in water and acetone, sparingly soluble in ethanol and in cold propylene glycol, but soluble in diethyl-ether, in petroleum, and in hot propylene glycol.

RESULTS

TABLE III

IDENTIFICATION OF PRODUCTS ISOLATED

Description	Results of Infra-red Analysis, etc.*
A. *Water soluble metabolites isolated after counter-current extraction*	
Stages 1 and 2: 21 mg. ether insoluble solids	Broad band at 1,100 cm.$^{-1}$ indicated possible hydroxylated acridine.
144 mg. hydrochloride from ethereal solution.	Mepacrine hydrochloride.
Stages 3, 4 and 5: 480 mg. of hydrochloride isolated.	Mepacrine hydrochloride, containing from 5–10% of an unknown compound resembling that from stage 6.
Stage 6: 7 mg. ether insoluble base.	Possibly mepacrine metabolite having considerably modified side chain.
B. *Petroleum soluble metabolites isolated after passage down a "Decalso" column.*	
Fraction 1: 83 mg. total solids yielding 12 mg. after recrystallization from *n*-hexane.	8-chloro-3 : 5-dimethoxyacridine (m.p. 157°).†
Fraction 2: 144 mg. hydrochloride precipitated from ether.	Mepacrine hydrochloride.
4·5 g. of oil from the petroleum layer yielding 0·202 g. white crystals.	Crystals having m.p. 147° and free from nitrogen, sulphur and phosphorus.
Fraction 3: 106 mg. hydrochloride precipitated from ether.	Mepacrine hydrochloride.
Fraction 4:	Not identified.
Fraction 5: 20 mg. hydrochloride from top of column.	Mepacrine hydrochloride.

* The conclusions on the i.r. spectra are based on comparison with the spectra of mepacrine analogues bearing modified side chains. Several of the latter are formally analogous to metabolites identified by Titus *et al.* (1948) in metabolic studies of 4-aminoquinoline antimalarials.

† 8-chloro-3 : 5-dimethoxyacridine (m.p. and mmp. 157°) arose by interaction of mepacrine with methanol during the course of isolation, even although the conditions were very mild. *iso*-Propanol was used later as a less reactive alternative. 8-chloro-3 : 5-dimethoxyacridine is a weak base, is not extracted from petroleum by aqueous buffer of *p*H higher than 2, and is distinguished by a brilliant violet fluorescence even in non-polar solvents.

Identification of Acid Phosphatides as Mepacrine Binding Agents

It was noted above that whereas mepacrine base was strongly adsorbed from petroleum solution on to decalso, the crude petroleum extract from the livers of treated mice was developed into several bands or fractions. Also, although mepacrine could not be extracted from the crude product before column treatment either with *p*H 6 buffer or with 0.1N-HCl, yet mepacrine was readily extracted by *p*H 6 buffer from the main petroleum fractions 2, 3, and 5, eluted from the column. This behaviour could be explained by postulating a petrol-soluble organic acid salt of mepacrine base which, although

initially stable on treatment with dilute hydrochloric acid, exchanged mepacrine cation for sodium on passage down the decalso column (decalso is, in fact, an ion-exchange medium), so that the free mepacrine base subsequently eluted would be readily extracted with *p*H 6 buffer solution. The hypothesis was supported by the following observations: (i) when 340 mg. of the oily residue from the fraction 2b (Table II, the main acridine fraction) was dissolved in hexane, shaken with 1N-HCl, then washed with water, it had regained the ability to retain mepacrine base (10 mg.) against the extractive action of aqueous 0.1N-HCl solution. The object of acid washing the oil is to replace the metal cation picked up from the column by hydrogen. (ii) Potentiometric titration of 2b after acid treatment was carried out using a micro-technique. The titration curve was that of a weak acid (15.5 g.≡1 m-equiv. NaOH). The amount of oil which retained 1 m-equiv. of mepacrine base (m.w. 400) against extraction with 0.1N-HCl was of a similar order (13.6 g.).

Since a number of higher fatty acids (tested both as individuals and as mixtures obtained after saponification of linseed and castor oils), yeast nucleic acid and tauroglycocholic acid had no mepacrine binding power, it was decided to look for an appropriate fraction in the livers of undosed mice. After isolation as described above the product had a mepacrine binding power comparable with the oil isolated from the chromatographic column fraction 2b, and it contained phosphorus and nitrogen. The presence of phosphorus indicated that acidic phospholipids might be responsible for the effect, and in fact, when soya-bean lecithin in petroleum solution was shaken with 2N-HCl solution, then washed acid-free, the product had marked mepacrine binding power. As there are a number of phospholipids in this lecithin, the affinity of the acid phosphatides for mepacrine was more particularly investigated by preparing the phosphatidyl serine and inositol phosphatide fractions from ox-brain (Folch, 1942). After shaking with 2N-HCl and washing free from acid, approximately 80 mg. of these fractions in petroleum bound 10 mg. of mepacrine base against extraction with 0.1N-HCl. The addition of the phospholipids to mepacrine base in petroleum produced a colour change from very pale yellow to orange, which suggested salt formation.

In order to obtain an indication of the acid radical in the phosphatide obtained from the control mouse liver, part of the hexane solution was refluxed for three hours with 5N-HCl. The lower layer was removed, washed with hexane and then evaporated to dryness. Water was added to the residue, which was again later evaporated to dryness to remove hydrochloric acid. Paper strip chromatography of the residue developed with butanol-propanol-water (47 : 28 : 25 parts by volume) resulted in a spot reacting with ninhydrin and having an R_F value similar to that of a serine control.

Discussion

It has been shown above that when mice are given mepacrine hydrochloride (10 mg./mouse) by mouth, and a suitable period (48 hours) allowed for metabolism, approximately 94% of the total acridine in the livers was recovered as mepacrine. The remaining 6% was presumed to be metabolites (Table IV, c, d, and e ; b is an artefact). If these had contained a highly active constituent an antiviral effect should have been demonstrable from a small parenteral dose. However, at a level of 0.1 mg. b.i.d. for 5 days no activity was apparent, whereas mepacrine at 0.5 mg. b.i.d. for 5 days showed marked activity. It would appear, then, that the metabolites isolated do not contribute materially to the total antiviral effect.

A considerable proportion (26–38% according to the preparation) of the mepacrine was isolated in the form of acid phosphatide salts. These are soluble in fat solvents, insoluble in water, and not

Table IV

BIOLOGICAL TESTS

Mice (18–20 g.) were given drug by mouth and then infected with Eastern equine encephalomyelitis. The numerals in parentheses indicate the mean period of survival in days.

Ref.	Compound	Dose (g.) and Route	Deaths/20
	Control		16 (4·7)
	Mepacrine	10 oral once	0
		0·5 i.p. once	18 (6·0)
		0·5 i.p. b.i.d. 5 days	8 (6·1)
a	Mepacrine phosphatide	0·5 i.v. once	17 (5·5)
		0·5 i.v. b.i.d. 5 days	18 (4·7)
		0·5 i.p. once	18 (6·8)
		0·5 i.p. b.i.d. 5 days	12 (6·3)
	Control		16 (5·6)
	Mepacrine	10 oral once	3 (9·6)
b	8-Chloro-3 : 5-dimethoxy-acridine	10 oral once	14 (5·7)
		10 oral in oil	16 (6·2)
		5 i.v.	18 (5·5)
	Control		9 (5·0)
	Mepacrine	0·1 i.p. b.i.d. 5 days	7 (6·6)
c	Ether insoluble solids (Table IIIA stages 1 and 2)	0·1 i.p. b.i.d. 5 days	9 (4·9)
d	Ether insoluble base (Table IIIA stage 6)	0·1 i.p. b.i.d. 5 days	10 (5·4)
	Control		9 (5·7)
	Mepacrine	0·1 i.p. b.i.d. 5 days	10 (5·6)
e	Secondary component from stage 3–5, Table IIIA	0·1 i.p. b.i.d. 5 days	10 (5·7)

readily decomposed by dilute hydrochloric acid. It is quite probable that the mepacrine phosphatides are formed within the tissue, since, in preliminary work, acid stable, solvent soluble, mepacrine derivatives were not found after homogenizing mouse liver with mepacrine. These salts presumably account for the persistent staining noted in liver and other organs. This leads naturally to the idea of a depot preparation, but the injection of mepacrine diphosphoinositide (0.5 mg., i.p., b.i.d. 5 days) did not lead to promising results.

Summary

1. Mice were given mepacrine hydrochloride by mouth. After 48 hours, the animals were killed and the livers examined for metabolites. Of the acridine extracted, 94% was mepacrine ; the remainder was separated into three distinct fractions by countercurrent distribution methods. The fractions contained metabolites having infra-red spectra comparable to those of model compounds in which the side chain had been modified. The metabolites had no significant biological activity against Eastern equine encephalomyelitis in the mouse (after i.p. injection).

2. From 26 to 38% of the mepacrine isolated was in the form of phosphatide salts, which are very soluble in fat solvents and are not decomposed by dilute hydrochloric acid. The persistence of mepacrine in tissue is probably due in part to the formation of these salts with the cell lipids. Yet it is not possible to attribute depot effects to these salts, since the mepacrine salt of an acidic phosphatide prepared from ox-brain was not superior to mepacrine hydrochloride as an antiviral drug in the mouse.

The author thanks Mr. St. C. Flett for the infra-red analyses and Dr. E. Weston Hurst and P. Melvin for biological tests.

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THE ANTIVIRAL ACTIVITY OF ACRIDINES IN EASTERN EQUINE ENCEPHALOMYELITIS, RIFT VALLEY FEVER AND PSITTACOSIS IN MICE, AND LYMPHOGRANULOMA VENEREUM IN CHICK-EMBRYOS

BY

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(RECEIVED MARCH 17, 1956)

Previous reports (Hurst, Melvin, and Peters, 1952; Hurst, Peters, and Melvin, 1952) described the prophylactic and therapeutic activity of mepacrine against some of the smaller viruses infecting mice. This activity was not shared by a limited number of other acridines, some of which were fairly closely related to mepacrine, or by chloroquine or pamaquin in which the basic side-chain of mepacrine is attached to the quinoline nucleus. A later report (Hurst, Snow, and Roberts, 1955) dealt with the basiphil particles deposited in the liver and some other organs and tissues following administration of mepacrine, particles which had already been observed by Siegel and Mushett (1944) and by Fitzhugh, Nelson, and Calvery (1945). The details of formation and distribution of these particles differed somewhat in other animal species (guinea-pig, rat, rabbit, chick, monkey, and dog), and in a given species no correlation existed between their formation and the presence of a therapeutic effect against equine encephalomyelitis. The therapeutic effect was outstanding only in the mouse, and apparently lacking in several animal species.

Collaterally with these studies we examined other acridines for therapeutic activity, and for the ability to give rise to basiphil particles, in the mouse. Because of the effect of certain nitroacridines on the largest viruses (Eaton, van Allen, and Wiener, 1947; Hurst, 1948), we also examined a number of compounds for activity in psittacosis and lymphogranuloma venereum. A few were tested against Rift Valley fever.

METHODS

Since the effect of a single dose of mepacrine (10 mg./20 g.; but see footnote Hurst, Snow, and Roberts, 1955) given near the time of infection with equine encephalomyelitis virus is almost as good as that of twice-daily doses of the drug, and since we were seeking a degree of activity comparable to or greater than that of mepacrine, we normally administered a single oral dose of the various compounds 24 hr. before the virus. Where possible we gave 10 mg./20 g., but at times, owing to toxicity, we had to be content with smaller doses. Sometimes we studied also the effect of twice-daily oral doses over a period of 6 or more days. At times, with compounds known to be very sparingly soluble, we had recourse to intravenous injection; or, rarely, to dispersion in oil, instead of in an aqueous medium, in the hope of promoting absorption from the alimentary canal. Other details of technique, including the choice of mice, and the maintenance of virus, have been discussed in an earlier paper (Hurst, Melvin, and Peters, 1952).

The course of development of macroscopic coloration in the tissues of mice treated with mepacrine, and of histological changes in the liver and elsewhere, suggested that 48 hr. after administration of the compound was a suitable time for detecting "persistent" coloration and basiphil particles, and accordingly we examined treated, uninfected animals after this interval. Examination of frozen-dried tissue never revealed basiphil particles when they had not been detected after conventional methods of processing the tissues.

The experiments with lymphogranuloma and psittacosis employed viruses and techniques described previously (Hurst, Peters, and Melvin, 1950).

RESULTS

Equine Encephalomyelitis

The first indication that other acridines might possess therapeutic activity against equine encephalomyelitis came from discussions with Professor A. Albert, who very kindly made available two compounds for which he predicted activity. These compounds, "Nor-atabrine" [2-chloro-5-(4-diethylaminobutylamino)-7-methoxyacridine+2HCl+$3H_2O$] and "Acranil" [2-chloro-5-(3-diethylamino-2-hydroxypropylamino)-7-methoxyacridine dihydro-

chloride],* differ from mepacrine only in a slightly modified basic side-chain. Both, and especially acranil, appeared to be more toxic than mepacrine, in that they caused more loss of weight and condition in treated mice. Both, however, showed therapeutic activity against equine encephalomyelitis. Treatment with acranil led to an appreciable reduction in mortality associated with an increased mean-survival-time of the animals that died; the action of nor-atabrine was mainly confined to increasing survival-time. Both drugs caused "persistent" macroscopic coloration of the tissues, and the livers contained abundant basiphil particles which with acranil were larger and far more conspicuous than with mepacrine.

Other compounds since examined are listed in Table I. Here, to make the data from many experiments more manageable, we have expressed the therapeutic activity as a percentage of that of mepacrine *in the same experiment*. If treatment with mepacrine resulted in a sparing of 16 lives as compared with the untreated controls, and treatment with another compound a sparing of 8 lives, the latter was deemed to possess an activity of 50%. This method of computation does not take into consideration the mean period of survival of the animals which died, but major effects—60% or more—are almost always accompanied by a correspondingly increased mean-survival-period, while appreciably lesser effects are often not reproducible.

The following information will assist in evaluating the results of treatments with less than the normal dose of 10 mg./20 g. After a single oral dose of 10 mg./20 g. mepacrine given 24 hr. before virus, 0 to 3, or seldom 4 or more, mice die in a group of 20, whereas among the controls 16 to 19, or sometimes 15 or 20, animals die. A dose of 5 mg./20 g. in 11 consecutive experiments gave effects varying from 56 to 109%, with a mean of 89%; a dose of 2 mg./20 g. gave a mean of 64% in five experiments; and a dose of 1 mg. gave 36 and 31% in two experiments.

Table I shows that a number of acridines other than mepacrine possess considerable activity against equine encephalomyelitis in mice. In the order in which they appear in the table, these are 16,469, 16,468, 5,770, 10,064, 15,431, 17,465, 16,470, and 16,688. Where tested by administration of a range of doses, none appeared significantly more active than mepacrine. All these compounds produced "persistent" coloration of the tissues and gave rise to basiphil particles in the liver, usually to about the same extent as mepacrine. Sometimes, however, as with 16,468, macroscopic coloration was much more intense than with mepacrine, and the basiphil particles were far coarser and more numerous; in the liver cells they frequently attained a diameter of 2 μ, and in the Kupffer cells of 9 μ. Doses of this compound larger than 5 mg. caused extensive necrosis of the liver; the dead cells rarely contained any basiphil material, indicating both their rapid destruction and the primary involvement of the Kupffer cells noted previously (Hurst, Snow, and Roberts, 1955).

A second group of compounds (15,430, 16,570, and 16,527) apparently possessed less therapeutic activity, but coloured the tissues and produced basiphil particles. A third group (15,243, 16,372, 15,541, and 16,286), showing about the same apparent activity as the second group, seemed to differ in their inability to stain the tissues and to produce basiphil particles.

While, therefore, major activity seemed always to be accompanied by these pigmentary changes, lesser degrees of activity, if genuine, were not necessarily so associated. Conversely, with 17,359 an appreciable tendency to produce pigmentary change was not accompanied by a convincing therapeutic effect. The behaviour of 6,769 will be mentioned below.

None of a series of acridans or acridones possessed activity; neither did three dialkylaminoalkylamines nor several quinolines (including chloroquine, previously found devoid of activity by Hurst, Peters, and Melvin, 1952).

Rift Valley Fever

A few of these compounds were examined for activity against the virus of Rift Valley fever; against this infection acranil, 16,469, 16,470, and 16,688 showed activity; nor-atabrine, 16,468 and 5,770 were probably active; and 15,517, 16,472, 15,810, and 15,518 were inactive. These results agree well with those in equine encephalomyelitis.

Lymphogranuloma Venereum and Psittacosis

The maximum tolerated dose of many acridines given intravenously or intraperitoneally to mice is a mere fraction of that accepted orally, and sometimes is below that which experience would suggest as likely to be needed for therapeutic activity against equine encephalomyelitis. Similarly, many are poorly tolerated by the developing chick-embryo. Apparent lack of activity against lymphogranuloma virus in the embryo may therefore reflect only the inadequacy of the dose that can be administered. The following compounds showed no appreciable activity when a single dose was given 2½ hr. following virus into the yolk-sac (the figures in parentheses

* In this, as opposed to earlier papers, we use the nomenclature recommended by the Chemical Society.

TABLE I

EXAMINATION OF VARIOUS ACRIDINES, ETC., FOR ACTIVITY AGAINST EASTERN EQUINE ENCEPHALITIS IN MICE

Single doses were given, or twice-daily treatment begun, 24 hr. before virus in groups of 20 mice. The results are expressed as percentages of those produced by 10 mg. mepacrine *in the same experiment* (for details see text). A single asterisk indicates a significant therapeutic effect at the $P=0.05$ level, as calculated by means of the tables of Loewenthal and Wilson (1939). A double asterisk indicates a significant effect at the $P=0.005$ or lower levels. The percentages needed to establish a significant difference from the effect of mepacrine varied, in the different experiments, from 33 to 50 ($P=0.05$) or 50–64 ($P=0.005$). (S) = increased mean period of survival of fatal cases exceeding that in controls by more than one day.

Cmpd. No.	Name of Compound	Dose (mg./20 g.) and Route	Therapeutic Effect (Mepacrine = 100)
	Acridines		
—	Acridine	20 oral 20 × 2 oral	−6, 0 11
15,243	5-Benzylacridine	20 × 1 i.v. 10 oral 10 ,, (in oil)	−13, 0 60, 27 (S) 23
15,281	5-(2-Piperidinoethyl)-acridine dihydrochloride	10 oral	17, −20
—	5-Aminoacridine (" Aminacrine ")	1 ,, 12 × 0·5 oral	17, 39, −21 −33, 17, 22, −14
16,256	5-[3-(2-Hydroxyethylamino)-propylamino]-acridine dihydrochloride	10 oral 5 ,, 2 ,,	0 30 7
18,006	5-(4-Diethylamino-1-methylbutylamino)-acridine dihydrochloride	5 ,,	13, −6
11,535	5-Acridone	4 i.v. 12 × 2 i.v.	−13, −36 −13, −25
11,536	Acridan	10 oral 1 i.v.	0 −6
15,244	5-Benzylacridan	10 oral	−10, 20
15,543	Sodium acridan-5-sulphonate	10 ,,	20, 0
15,468	1-Methoxyacridan	10 ,, 1 i.v.	0 0
7,706	5-Amino-3-methoxyacridine	10 oral	22
15,372	5-[2-(2-Hydroxyethylamino)-ethylamino]-3-methoxyacridine dihydrochloride	10 ,,	−20,27 (S)
15,517	5-[2-(2:3-Dihydroxypropylamino)-ethylamino]-3-methoxyacridine dihydrochloride	10 ,, 12 × 5 oral	−20, 13 20
16,469	5-(2-Diethylaminoethylamino)-3-methoxyacridine dihydrobromide	10 oral 5 ,,	100 (S)** 88 (S)**
16,115	5-[3-(2-Hydroxyethylamino)-propylamino]-3-methoxyacridine dihydrochloride	10 ,,	17 (S)
16,468	5-(3-Diethylaminopropylamino)-3-methoxyacridine dihydrobromide	5 ,, 2 ,, 1 ,,	50 (S)* 50 (S)* 31 (S)
16,472	5-(2-Diethylaminoethylmethylamino)-3-methoxyacridine dihydrobromide	10 ,,	25
12,642	3-Methoxyacridan	10 ,, 1 i.v.	13 0
16,287	3-Butoxy-5-[3-(2-hydroxyethylamino)-propylamino]-acridine dihydrochloride	5 oral	−29
17,236	2-Chloro-7-methoxyacridine	10 ,, 1 i.v.	6 13
11,838	2: 5-Dichloro-7-methoxyacridine	10 oral	0
15,810	2-Chloro-5-*p*-dimethylaminophenyl-7-methoxyacridine	10 ,,	6, 0
17,381	2-Chloro-5: 7-dimethoxyacridine	10 ,, 10 ,, (in oil) 5 i.v.	15 0 −15
17,213	2-Chloro-5-ethoxy-7-methoxyacridine	10 oral	19
17,548	2-Chloro-7-methoxy-5-thiolacridine	10 ,, 12 × 10 oral	−27 −18

TABLE I—*continued*

Cmpd. No.	Name of Compound	Dose (mg./20 g.) and Route	Therapeutic Effect (Mepacrine = 100)
17,754	S-(2-Chloro-7-methoxy-5-acridinyl)-aminothiuronium chloride	10 oral 12 × 2 5 oral	−45 −18
17,534	S-(2-Chloro-7-methoxy-5-acridinyl)-thioglycollic hydrazide	10 oral	9
4,020	5-Amino-2-chloro-7-methoxyacridine	10 ,,	31, 22 (S), 0
11,722	2-Chloro-7-methoxy-5-methylaminoacridine acetate	10 ,, 12 × 2 oral	−6, −36 −13, −17
11,721	2-Chloro-7-methoxy-5-*iso*propylaminoacridine acetate	20 oral 12 × 2 oral	−50* −6, −33
11,743	5-*n*Butylamino-2-chloro-7-methoxyacridine acetate	10 oral 12 × 2 oral	−42 −33
16,740	2-Chloro-5-(2: 3-dihydroxypropylamino)-7-methoxyacridine	10 oral	0
16,525	N-(2-Chloro-7-methoxy-5-acridinyl)-β-alanine	10 ,,	−14
15,430	2-Chloro-5-[2-(2-hydroxyethylamino)-ethylamino]-7-methoxyacridine dihydrochloride	10 ,,	40, 33 (S)
16,372	2-Chloro-5-[2-(2: 3-dihydroxypropylamino)-ethylamino]-7-methoxyacridine dihydrochloride	10 ,,	20, 47 (S)*
16,897	2-Chloro-5-(2-benzylaminoethylamino)-7-methoxyacridine dihydrochloride	10 ,,	14
15,432	2-Chloro-5-[3-(2-hydroxyethylamino)-propylamino]-7-methoxyacridine dihydrochloride	10 ,,	60, 40*
16,895	2-Chloro-5-[3-(2-hydroxyethoxy)-propylamino]-7-methoxyacridine hydrochloride	10 ,,	−14
5,770	2-Chloro-7-methoxy-5-(3-piperidinopropylamino)-acridine dihydrochloride	10 ,, 5 ,, 2 ,, 1 ,,	86 (S)**, 64 (S)** 93 (S)** 57* 29
4,160	2-Chloro-5-dimethylamino-7-methoxyacridine	10 ,, 2 i.v. 12 × 2 i.v.	−13, 0 0, 0 0
7,337	2-Chloro-5-piperidino-7-methoxyacridine	10 oral 2 i.v. 12 × 1 i.v.	0, 0 −13, 0
15,156	2-Chloro-7-methoxy-5-*p*-methoxycarbonylanilinoacridine	10 oral 12 × 5 i.p.	−30, 0 0
11,742	2-Diethylaminoethyl-*p*-(2-chloro-7-methoxy-5-acridinylamino)-benzoate dihydrochloride	20 oral 12 × 5 oral	11, −25 17
15,187	*p*-(2-Chloro-7-methoxy-5-acridinylamino)-benz-(4-diethylamino-1-methylbutyl)-amide dihydrochloride	10 oral	40*, 10
10,064	2-Chloro-5-(4-diethylamino-1-methylbutylamino)-7-methoxyacridine di-*N*-oxide dihydrochloride (" Mepacrine di-*N*-oxide ")	10 ,, 5 ,, 2 ,, 1 ,,	65*, 88 (S)** 50 (S) 6 (S) 0
18,959	2: 5-Dichloro-7-hydroxyacridine	10 ,,	−13
19,178	2-Chloro-5-(4-diethylamino-1-methylbutylamino)-7-hydroxyacridine citrate	10 ,,	13
1,616	2-Chloro-7-methoxyacridone	10 ,,	−20
13,622	2-Chloro-7-methoxyacridan	10 ,, 2 i.v.	0 6
17,028	5-Amino-3-chloro-7-methoxyacridine	10 oral	25, 6 (S), 7
16,687	N-(3-Chloro-7-methoxy-5-acridinyl)-β-alanine	10 ,,	−14
15,431	3-Chloro-5-[2-(2-hydroxethylamino)-ethylamino]-7-methoxyacridine dihydrochloride	10 ,,	60, 66(S)**
15,518	3-Chloro-5-[2-(2: 3-dihydroxypropylamino)-ethylamino]-7-methoxyacridine dihydrochloride	10 ,, 12 × 5 oral	11, −60* −13
15,541	3-Chloro-5-[3-(2-hydroxyethylamino)-propylamino]-7-methoxyacridine dihydrochloride	5 oral	60, 33

TABLE I—*continued*

Cmpd. No.	Name of Compound	Dose (mg./20 g.) and Route	Therapeutic Effect (Mepacrine = 100)
16,570	3-Chloro-5-(3-ethylaminopropylamino)-7-methoxyacridine dihydrochloride	5 oral	36 (S)
15,809	3-Chloro-7-methoxyacridan	10 ,, 1 i.v.	−6 0
17,465	5-(4-Diethylamino-1-methylbutylamino)-3-methoxy-7-nitroacridine	10 oral 5 ,, 2 ,, 1 ,,	87 (S)**, 82 (S)** 108 (S)** 64 (S)* 45
17,466	3-Amino-5-(4-diethylamino-1-methylbutylamino)-7-methoxyacridine trihydrochloride	10 ,,	9 (S), 6
18,170	5-Chloro-3-methoxy-8-nitroacridine	10 ,,	−12
19,198	5-(4-Diethylamino-1-methylbutylamino)-3-methoxy-8-nitroacridine	5 ,,	0
17,689	5-Chloro-2: 3-dimethoxy-8-nitroacridine	10 ,,	−27, −19
6,769	5-(3-Diethylamino-2-hydroxypropylamino)-2: 3-dimethoxy-8-nitroacridine	5 ,, 12×2 oral 6×0·25 i.p.	25, −6 (S) 0 0
18,731	5-(4-Diethylamino-1-methylbutylamino)-2: 3-dimethoxy-8-nitroacridine dihydrochloride	10 oral	−20
17,359	2-Chloro-5-(4-diethylamino-1-methylbutylamino)-7-*p*-nitrobenzyloxyacridine	10 ,, 5 ,, 12×1 oral	17, 0 (S) 9 (S) −9 (S)
16,286	2-Chloro-5-[3-(2-hydroxyethylamino)-propylamino] acridine dihydrochloride	10 oral	50, 40 (S)*
15,875	2-Chloroacridan	10 ,, 1 i.v.	−6 −6
15,876	3-Chloroacridan	10 oral 1 i.v.	6 0
10,997	3-Chloroacridone	10 oral 1 i.v.	0 6
16,527	2: 7-Dichloro-5-[3-(2-hydroxyethylamino)-propylamino]-acridine dihydrochloride	10 oral	36 (S)
16,741	1: 4-Dichloro-5-[3-(2-hydroxyethylamino)-propylamino]-acridine dihydrochloride	10 ,,	0
16,473	1: 3-Dichloro-5-(2-diethylaminoethylamino)-acridine dihydrobromide	10 ,,	50 (S)*, 0 (S), 17 (S)
16,470	1: 3-Dichloro-5-(3-diethylaminopropylamino)-acridine dihydrobromide	10 ,, 5 ,, 2 ,, 1 ,,	100 (S)**, 121 (S)** 121 (S)** 50 (S)* −20
16,688	1: 3-Dichloro-5-[3-(2-hydroxyethylamino)-propylamino]-acridine dihydrochloride	10 ,, 5 ,, 2 ,, 1 ,,	100 (S)**, 107 (S)** 79 (S)** 50 (S)* 0
18,044	1: 3-Dichloro-5-(4-diethylamino-1-methylbutylamino)-acridine dihydrobromide	2 ,,	0 (S), 0
10,996	1: 3-Dichloroacridone	10 ,,	0
15,698	3: 7-Dimethylacridan	10 ,, 2 i.v.	19, −13 6, −7
17,006	Diacriflavine	10 oral 2·5 ,,	−6 0, 0
	Euflavine	5 ,, 12×0·5	11, 17, 14 −33, 6, 17,
	Proflavine	oral	0
17,650	Polymer from proflavine dihydrochloride and hexamethylene-*bis*-dicyandiamide	10 oral	−27
	Quinolines		
15,157	7-Chloro-4-*p*-carboxyanilinoquinoline	12×2 i.p.	0
16,569	7-Chloro-4-[3-(2-hydroxyethylamino)-propylamino]-quinoline	10 oral	0
	Chloroquine	10 ,, 12×1 oral 12×0·5 i.p.	0 −11, −33 −11, 7, 24

TABLE I—*continued*

Cmpd. No.	Name of Compound	Dose (mg./20 g.) and Route	Therapeutic Effect (Mepacrine = 100)
5,120	4-(3-Diethylaminopropylamino)-6-methoxy-3-methylquinoline dihydrochloride	10 oral 12×2 oral	−20 −13
5,372	4-(3-Diethylaminopropylamino)-2: 3-dimethylquinoline sulphate	10 oral	40 (S)*, 11, 10, 19
16,965	4: 4′-(Ethylenedioxy)-*bis*-7-chloroquinoline	5 ,,	0
16,966	7-Chloro-4-(2-ethoxyethoxy)-quinoline	10 ,,	−6
	Amines		
3,857	4-Diethylamino-1-methylbutylamine	5 ,,	−20
16,917	*N*-(4-Diethylamino-1-methylbutyl)-guanidine sulphate	10 ,,	0
17,104	*N*-(4-Diethylamino-1-methylbutyl)-benzylamine	10 ,, 5 ,,	25, 6 −14

are the larger doses (in mg.) employed; in addition each compound was tested at one-quarter or one-third of this dose):

7,337 (0.2), 10,064 (0.5), 11,742 (0.2), 15,156 (0.5), 15,187 (1.25), 15,243 (10), 15,244 (10), 15,281 (2.5), 15,372 (0.1), 15,430 (0.5), 15,431 (0.5), 15,541 (0.2), 16,115 (0.05), 16,256 (0.05), 16,286 (0.05), 16,527 (0.05), 16,570 (0.5), 16,687 (1.25), 16,740 (0.5), 16,741 (0.5), 17,359 (0.2), 17,465 (0.2), 17,466 (1.25), 17,534 (0.1), 17,548 (0.05), 17,689 (2.5), 17,754 (0.05), 18,006 (0.2), 18,044 (2.5), 18,170 (0.01), 18,959 (0.5), 19,178 (0.5), 19,198 (0.5).

In all tests mepacrine itself was included and was always without appreciable effect. However, one of the compounds listed above (19,198) possessed definite activity in the mouse but none in the chick-embryo.

Of compounds available in sufficient quantity for test against psittacosis in mice, the following showed no activity when given at the dose stated, twice daily for 12 days, beginning 4 hr. before virus:

7,337 (5 mg. oral), 11,742 (5 mg. oral), 15,156 (5 mg. i.p.), 15,430 (5 mg. oral), 15,431 (2 mg. oral), 16,687 (5 mg. oral), 17,466 (2 mg. oral), 17,754 (2 mg. oral), 18,959 (5 mg. oral), 19,178 (2 mg. oral).

Euflavine, proflavine, and mepacrine were also inactive. As shown in Table II, three compounds had considerable activity. Compound 6,769—which, as "Nitroakridin 3582," was previously shown by Eaton, van Allen, and Wiener (1947), and by Hurst (1948), to possess therapeutic properties against the largest viruses—was now administered orally and in larger doses than heretofore. Compound 18,731 was a similar compound with the mepacrine side-chain, and 19,198 an $-NO_2$ analogue of mepacrine itself. Of these, the second compound was less toxic than the others and appeared to be the best, both when judged by the mortality in treated animals and by the clinical condition of the mice during the period of therapy. All these compounds were active when dosing was deferred until 48 hr. after infection, and with 18,731 the clinical condition of the mice was nearly as good

TABLE II

ACTION OF THREE NITROACRIDINES AGAINST PSITTACOSIS IN MICE

Virus was given intraperitoneally, drug orally. The figures in parentheses are the mean periods of survival of fatal cases in days. Compound 6769 is identical with "Nitroakridin 3582" prepared originally by A. G. Hoechst, Main.

No.	Name	Oral Dose (mg./20 g., b.i.d.)	Time of First Dose	Deaths in 10 Mice (i)	Deaths in 10 Mice (ii)
—	No drug given	—	—	9 (6·1)	9 (7·3)
—	Aureomycin	2	48 hr. after virus	0	0
6,769	5-(4-Diethylamino-2-hydroxypropylamino)-2: 3-dimethoxy-8-nitroacridine dihydrochloride	2	4 hr. before virus	1 (12·0)	2 (16·0)
		2	48 hr. after virus	—	2 (11·0)
18,731	5-(4-Diethylamino-1-methylbutylamino)-2: 3-dimethoxy-8-nitroacridine dihydrochloride	4	4 hr. before virus	0	0
		4	48 hr. after virus	—	0
19,198	5-(4-Diethylamino-1-methylbutylamino)-3-methoxy-8-nitroacridine	1	4 hr. before virus	0	3 (16·5)
		1	48 hr. after virus	—	3 (14·8)

as in those receiving aureomycin. With diminishing dosage, however, the activity of 18,731 fell off very much more rapidly than did that of aureomycin.

Under the conditions of treatment in Table I, 18,731 and 19,198 produced no "persistent" coloration of the tissues and no basiphil particles were detected in the liver 48 hr. after the dose; as we have seen, they were inactive against equine encephalomyelitis. The behaviour of 6,769 was slightly different. Forty-eight hr. after a single dose we noted no "persistent" coloration, but on histological examination a few basiphil flecks were present. Four hr. after the last of five twice-daily doses, however, the tissues were faintly yellow and abundant basiphil particles were seen in the liver and Kupffer cells; again only traces of the particles remained 48 hr. later. Apparently, therefore, this compound gives rise to basiphil material which is less durable than that produced by mepacrine. On retesting, both with a single large dose and with repeated smaller intraperitoneal doses (Table I), we confirmed our previous finding that the drug is not active against equine encephalomyelitis (Hurst, 1948). The factor determining activity against the largest rather than the smaller viruses is not the presence of the $-NO_2$ group, as appeared likely from the experiments of Eaton, van Allen, and Wiener, because 17,465, in which the $-NO_2$ group occupied position 7 instead of position 8 of the acridine nucleus, was quite highly active against equine encephalomyelitis. Although our experience with lymphogranuloma and psittacosis is clearly limited, we have not yet encountered a compound in which activity has been present simultaneously for both types of virus.

SUMMARY

1. Of 83 acridines examined, some showed therapeutic activity against equine encephalomyelitis in mice. The activity was never significantly greater than that of mepacrine. Major activity was always associated with "persistent" coloration of the tissues by the drug, and by the formation of basiphil particles. The converse did not hold. Minor apparent activity might or might not be associated with these phenomena. Three dialkylaminoalkylamine and seven quinoline derivatives had no beneficial effect.

2. The results of testing a few compounds against Rift Valley fever in mice agreed well with those in equine encephalomyelitis.

3. Many acridines are very poorly tolerated by the chick-embryo and cannot be administered in doses likely to be effective against lymphogranuloma venereum infections; 33 compounds were inactive against lymphogranuloma venereum in the yolk-sac.

4. Three nitroacridines were considerably active against psittacosis in the mouse, but were inactive against equine encephalomyelitis. The factor determining therapeutic activity against the largest or the smaller viruses is not the $-NO_2$ group, because one nitroacridine was active against equine encephalomyelitis.

5. We have not yet found a compound which is active against both small and large viruses.

Some of the acridines were kindly supplied by Drs. S. Birtwell, A. F. Crowther, P. B. Dransfield, G. W. Driver, P. B. Gaubert, R. R. Goodall, W. G. M. Jones, J. K. Landquist, G. Swain, R. A. Wilkinson, and B. Wilson.

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Printed in Great Britain by Fisher, Knight & Co., Ltd., Gainsborough Press, St. Albans, Herts.